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13. ABSTRACT ***(Maximum 200 Words)***

Advanced prostate cancer continues to kill 32,000 men per year in the United States. Despite strides in obtaining partial, temporary remissions in men with advanced disease through the use of hormones and chemotherapeutic agents, there is no curative therapy for advanced prostate cancer. Most prostate cancers are responsive to androgens, and androgen withdrawal (i.e., surgical or medical castration) is the main form of treatment for advanced (i.e. disseminated) disease.[1] The failure of primary hormone therapy is attributed to androgen-independent tumor expansion. Although the androgen receptor (AR) is present in these cells, it is nonfunctional.[2] These cells have been shown to grow by other means. Many androgen independent cells survive by acquired resistance to many cell death inducing factors. At this point, there is no clear explanation as to why prostate cancer cells develop androgen independence. We believe that a molecule called CBP [CREB (cAMP response element binding protein)-binding protein] may play a critical role. CBP associates with several other sequence-specific factors, including NF-κB and AR. Both NF-κB and AR require CBP for their activation. We investigated the effects of inhibitors of NF-κB as well as other chemotherapeutic agents to treat hormone refractory prostate cancer.

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Table of Contents

Introduction

Advanced prostate cancer continues to kill 32,000 men per year in the United States. Despite strides in obtaining partial, temporary remissions in men with advanced disease through the use of hormones and chemotherapeutic agents, there is no curative therapy for advanced prostate cancer. Most prostate cancers are responsive to androgens, and androgen withdrawal (i.e., surgical or medical castration) is the main form of treatment for advanced (i.e. disseminated) disease.[1] The failure of primary hormone therapy is attributed to androgen-independent tumor expansion. Although the androgen receptor (AR) is present in these cells, it is nonfunctional.[2] These cells have been shown to proliferate by other means, such as insulin-like growth factor (IGF)[3] and interleukin-6 (IL-6).[4] Many androgen independent cells survive by acquired resistance to many apoptotic factors, such as TNF-α.[5] At this point, there is no clear explanation as to why prostate cancer cells develop androgen independence. We believe that the CBP [CREB (cAMP response element binding protein)-binding protein] may play a critical role.

CBP is a transcriptional co-regulator that interacts with different DNA binding proteins and components of the general transcription machinery.[6] It is a bridging factor between CREB and the basal transcriptional apparatus.[7] In addition to CREB, CBP associates with several other sequence-specific factors, including NF-κB and AR.[8] Both NF-κB and AR require CBP for their transactivation.[6] However, CBP has a greater affinity for NF-κB than AR.[6] As demonstrated in our preliminary data, if NF-κB is active, AR is inactive. NF-κB has already been implicated in the negative regulation of the rat AR gene promoter.[9]

NF-κB is held in the cytoplasm by its inhibitor, IκBα. This retention is uncoupled by many extracellular signals, including TNF-α. Through a series of events, TNF-α causes phosphorylation of IκBα which rapidly gets degraded and releases NF-κB. Once released, NF-κB translocates to the nucleus, binds CBP, and begins transcription. We investigated the role of NF-κB in androgen sensitive and insensitive cells and then applied this to designing new therapies for prostate cancer.

Body

The original specific aims of our proposal were to:

Specific aim 1: Create stable cell lines of LNCaP (PSA-producing; androgen sensitive), PC-3 (non-PSA-producing; androgen insensitive), and C4-2B (PSA-producing; androgen insensitive) containing the PSA-IκBα "super repressor" construct.

Specific aim 2: Use animal models to demonstrate that PSA-IκBα "super-repressor" construct is stable and functionally effective in vivo.

Specific aim 3: Demonstrate that transfection of the PSA-IκBα "super-repressor" results in re-expression of a functional androgen receptor.

We were able to achieve specific Aim 1 and report that by transfecting cells with a IκBα super repressor, we were able to induce apoptosis in previously resistant cells, utilizing TNF-α.[10] The IκBα super repressor caused re-expression of the androgen receptor.[11] We then went on to investigate how the androgen receptor and apoptosis was related in androgen sensitive and

insensitive PC cells.[12] We found that androgen sensitive cells used a different caspase pathway than AI cells, suggesting that we may be able to develop better therapeutic strategies for different populations of cancer cells.[13, 14] We next demonstrated that stromal and cytokine components of the tumor microenvironment can effect sensitivity to androgen and behavior of the cancer cells.[15] Therefore, we have made several contributions to how androgens interact with AS and AI prostate cancer cells.

Key Research accomplishments

- The androgen receptor is still functional in AI cells, but that function is masked.
- Chemotherapeutic agents use different apoptotic pathways in AS and AI cells.

Reportable outcomes

The manuscripts and abstracts (appended) listed below reflect the work accomplished with the support of this award. In addition, this award served as a springboard to a tenure track position for Dr. Cooper as an Assistant Professor of Biological Sciences at the University of Delaware-Newark. Personnel who have received pay from this research effort are:
Heather Muenchen, Ph.D. – Investigator
Carlton R. Cooper, Ph.D. - Investigator
Kenneth J. Pienta, M.D. – Project Mentor

Manuscripts

Muenchen, H.J., Lin, D.L., Walsh, M.A., Keller, E.T., Pienta, K.J. Tumor necrosis factor-alpha-induced apoptosis in prostate cancer cells through inhibition of nuclear factor-kappaB by an I kappa B alpha "super repressor." Clin Cancer Res 6:1969-77, 2000.

Muenchen, H.J., Quigley, M.M., Pilat, M.J., Lehr, J.E., Brumfield, S.K., Mahoney, M., Pienta, K.J. The study of gemcitabine in combination with other chemotherapeutic agents as an effective treatment for prostate cancer. Anticancer Research 20:735-40, 2000.

Williams, J.F., Muenchen, H.J., Kamradt, J.M., Korenchuk, S., Pienta, K.J. Treatment of androgen-independent prostate cancer using antimicrotubule agents docetaxel and estramustine in combination: an experimental study. The Prostate 44:275-278, 2000.

Muenchen, H.J., Poncza, P.J., Pienta, K.J. Different docetaxel-induced apoptotic pathways are present in prostate cancer cell lines LNCaP and PC-3. Urology 57:366-370, 2001.

Cooper, C.R., Bhatia, J.K., Muenchen, H.J., McLean, L., Hayasaka, S., Taylor, J., Poncza, P.J., Pienta, K.J. The regulation of prostate cancer cell adhesion to human bone marrow endothelial cell monolayers by androgen dihydrotestosterone and cytokines. Clin Exp Metastasis 19:25-33, 2002.

Abstracts

Muenchen, H.J., Linn, D.L., Walsh, M.A., Keller, E.T., Pienta, K.J. Expression of clusterin in androgen independent prostate cancer cells after transfection with an IκBα "Super-Repressor." Proc AACR 41:4045, 2000.

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Conclusions

This has been a highly successful award that has resulted in key discoveries related to androgen receptor function and sensitivity to chemotherapy. Dr. Cooper used this award as a springboard to a tenure track position as an Assistant Professor of Biological Sciences at the University of Delaware-Newark.

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Tumor Necrosis Factor-α-induced Apoptosis in Prostate Cancer Cells through Inhibition of Nuclear Factor-κB by an IκBα "Super-Repressor"[1]

Heather J. Muenchen,[2] Din-Lii Lin, Michael A. Walsh, Evan T. Keller, and Kenneth J. Pienta

Department of Internal Medicine, Division of Hematology/Oncology [H. J. M., M. A. W., K. J. P.], Department of Animal Medicine, Division of Pathology, [D-L. L., E. T. K.], and Department of Surgery, Division of Urology [K. J. P.], University of Michigan, Ann Arbor, Michigan 48109

ABSTRACT

Prostate cancer patients experiencing a relapse in disease often express high serum tumor necrosis factor-α (TNF-α) levels. Many androgen-insensitive prostate cancer cells are TNF-α insensitive because of the expression of antiapoptotic genes as part of the nuclear factor-κB (NF-κB) family of transcription factors. NF-κB stimulates gene transcription when expressed in the nucleus; however, in resting cells, this nuclear import is prevented by association with the cytoplasmic inhibitor IκBα. This cytoplasmic retention of NF-κB is uncoupled by many extracellular signals including low levels of TNF-α. During normal cell activation, nuclear translocation of NF-κB is preceded by phosphorylation and degradation of IκBα. When phosphorylation is blocked, IκBα remains intact, thereby blocking NF-κB translocation to the nucleus and subsequent activation of antiapoptotic genes that cause TNF-α insensitivity. We tested whether a "super-repressor" of NF-κB activity could be transfected into prostate cancer cells and make them TNF-α sensitive. PC-3 and LNCaP cells were stimulated with TNF-α (10 ng/ml) for 24 h in the presence or absence of the IκBα "super-repressor" (p6R-IκB$_{S32A + S36A}$). NF-κB activity was measured by electrophoretic mobility shift assay and the steady state levels of the cytoplasmic IκBα protein were measured by Western blot. Secretory IL-6 and IL-6 mRNA were measured by ELISA. p6R-IκB$_{S32A + S36A}$ blocked the stimulation of NF-κB activity by TNF-α in prostate cancer cells. It also subsequently decreased IL-6 production by TNF-α. We conclude that these data demonstrate that inhibition of NF-κB selectively sensitizes previously insensitive prostate cancer cells to TNF-α.

INTRODUCTION

TNF-α[3] is known to possess potent antitumor activity both *in vivo* (1) and *in vitro* (2, 3). TNF-α is a polypeptide mediator of a variety of cellular responses, including apoptotic or necrotic cell lysis and proliferation (4, 5). It is predominately released from macrophages in response to foreign microbial components and has been established as an important mediator of tumor cell death, as demonstrated in previous experiments by others as well as the principal investigator and colleagues (3, 6–8).

TNF-α is not released from prostate cancer cells themselves but from associated macrophages after a relapse in disease (9). Studies have shown that patients with hormone-refractory prostate cancer demonstrate high serum TNF-α levels as compared with untreated patients (9–12). The relationship between TNF-α sensitivity and hormone responsiveness has not yet been explored. However, androgen-insensitive prostate cancer cells, PC-3 and JCA-1, have proven to be TNF-α insensitive, whereas androgen-sensitive prostate cancer cells, LNCaP, are TNF-α sensitive (13).

TNF-α triggers a number of signal transduction processes, which lead to either apoptosis or proliferation based on the TNF-α threshold of a cell. Most cells do not undergo apoptosis when exposed to low levels of TNF-α (Fig. 1*A*). This is likely a protective mechanism by the cell based on the induction by TNF-α of antiapoptotic genes such as A20, the Bcl-2 family member A1, manganese superoxide dismutase, and cellular inhibitor of apoptosis-2, all targets of the NF-κB family of transcription factors (14). It is this activation of antiapoptotic genes that makes cells insensitive to TNF-α-induced apoptosis. Apoptosis is induced by high levels of TNF-α binding to its receptor, TNF-RI, and activating members of the caspase family of proteases (Fig. 1*B*; Ref. 14). TNF-α-insensitive cells do not undergo TNF-α-induced apoptosis, regardless of the amount of TNF-α present (Fig. 1*C*). Instead, TNF-α causes constitutive expression of antiapoptotic genes protecting the cells from TNF-α-induced apoptosis.

Eukaryotic NF-κB is a well-established inducible transcription factor of great importance in cytokine-mediated inflammation (15). It is responsible for the rapid induction of many cytokines and adhesion molecules involved in the inflammatory and immune response systems (15, 16). Specifically, NF-κB participates in the activation of TNF-α-dependent ex-

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[3] The abbreviations used are: TNF-α, tumor necrosis factor-α; NF-κB, nuclear factor-κB; IL, interleukin; EMSA, electrophoretic mobility shift assay; PSA, prostate-specific antigen.

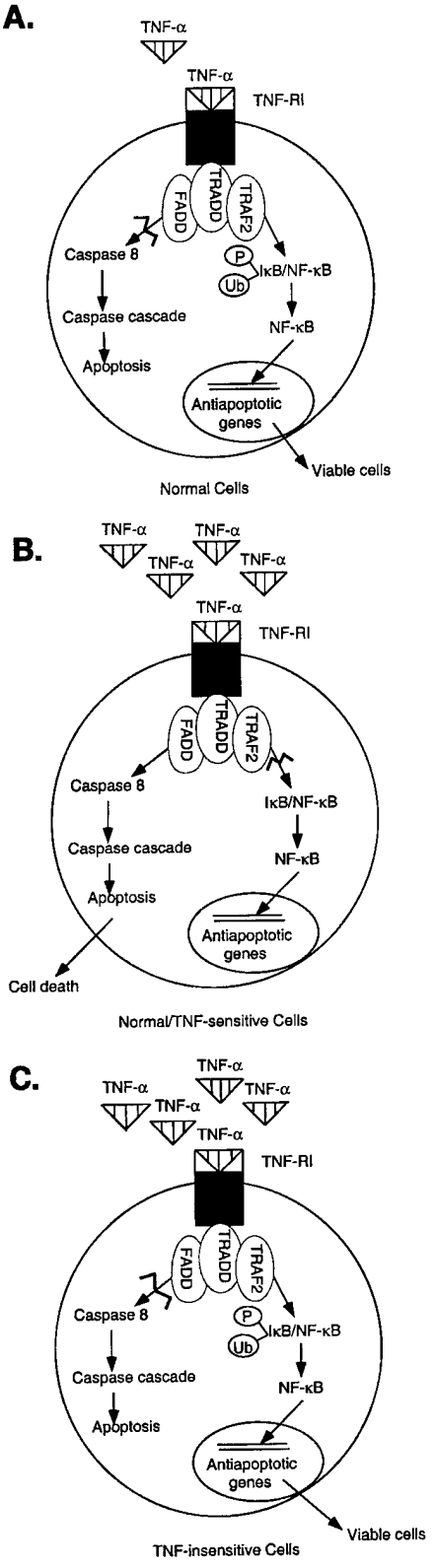

Fig. 1 Illustration demonstrating the differences between normal (*A*), normal/TNF-α-sensitive (*B*), and TNF-α-insensitive (*C*) cells. *FADD,* Fas-associated death domain; *TRADD,* TNFRI-associated death domain; *TRAF2,* TNF receptor-associated factor; *P,* phosphorylate; *Ub,* ubiquitinate.

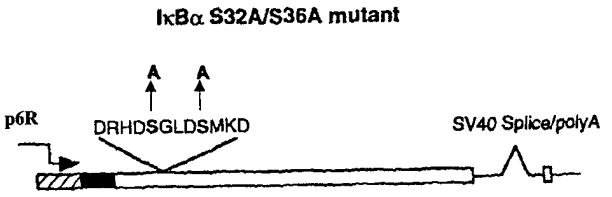

Fig. 2 Schematic representation of p6R-IκB$_{S32A + S36A}$ (18).

pression of IL-6, intercellular adhesion molecule-1, and matrix metalloproteinase-9, among others, in various cell systems (17, 18). IL-6, an inflammatory cytokine, is a known autocrine and paracrine growth factor in androgen-insensitive human prostate carcinomas (17). Intercellular adhesion molecule-1 is an established adhesion molecule involved in prostate cancer metastasis (17), whereas matrix metalloproteinase-9 plays an essential role in the destruction of the basement membrane because of its ability to proteolyze type IV collagen (18).

The prototypical form of NF-κB is a heterodimeric inducible complex containing two DNA binding subunits, p50 (NF-κB1) and p65 (RelA), both of which belong to the Rel family of transcription factors (19, 20). This heterodimer is the most potent gene transactivator within the NF-κB family (21). When NF-κB is expressed in the nucleus, it stimulates gene transcription via the potent transactivation domain located within the COOH-terminal half of RelA (22). However, in resting cells, the nuclear import of NF-κB is prevented because of a high-affinity association of its RelA subunit with a labile cytoplasmic inhibitor called IκBα (19, 23). This IκBα-dependent mechanism for the cytoplasmic retention of NF-κB is uncoupled by many extracellular signals including low levels of TNF-α (24). After this cellular stimulation, IκBα is phosphorylated at serines 32 and 36 by a specific kinase IKK (25), ubiquitinated, and undergoes proteolysis in proteosomes, enabling NF-κB to translocate to the nucleus, where it binds to NF-κB DNA binding sites and stimulates transcription of many cytokines, chemokines, and adhesion molecules (26).

A "super-repressor" form of IκBα with mutations at serines 32 and 36, located in the NH_2-terminal part of the polypeptide, has been shown to effectively prevent IκBα phosphorylation, degradation, and NF-κB activation in other systems (Fig. 2; Ref. 20, 27–30). With phosphorylation, blocked IκBα remains intact, thereby, blocking NF-κB translocation to the nucleus and subsequent activation of antiapoptotic genes that cause TNF-α insensitivity, thereby allowing the cells to proceed through apoptosis. This IκBα "super-repressor" has also been shown to induce apoptosis in other systems (31, 32). The "super-repressor" produces constitutive repression of NF-κB-directed transcription, despite the presence of agonists that normally induce the degradation of IκBα and the nuclear translocation of NF-κB (24).

Proteosome inhibitors such as calpain inhibitor I (ALLN) or MG-132 have been shown to block the induction of NF-κB activity in cultured cells (20). They inhibit IκBα degradation through their ability to block the catalytic activity of the proteosome complex (20). Although very effective in blocking the induction of NF-κB-activated antiapoptotic genes, proteosome

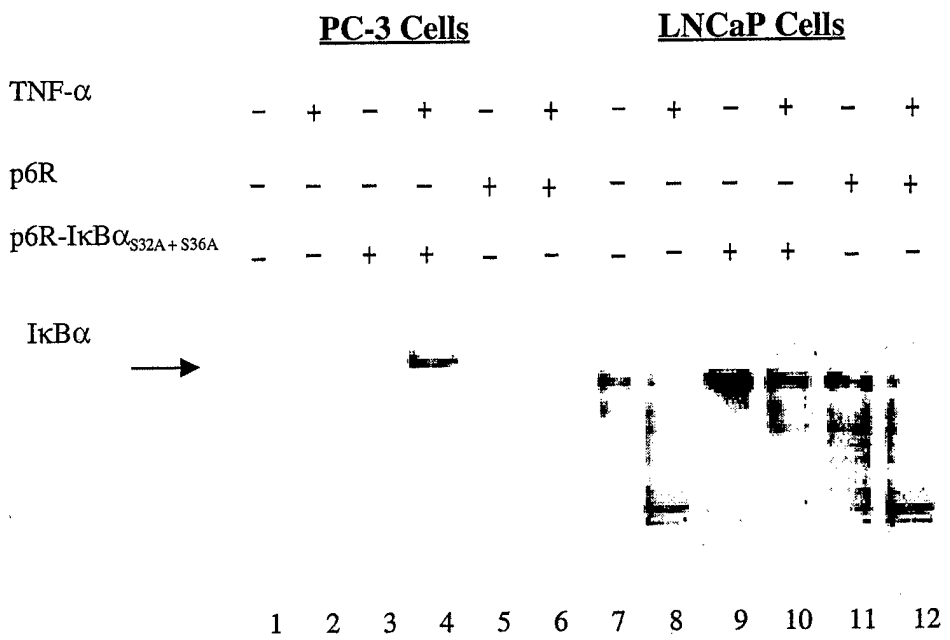

Fig. 3 The IκBα "super-repressor" blocks the degradation of IκBα by TNF-α in PC-3^T and LNCaP^T cells. PC-3 and LNCaP cells were first transfected with p6R-IκB$_{S32A + S36A}$ or the control p6R alone for 24 h before stimulation with 10 ng/ml of TNF-α for 24 h. Total cellular proteins (50 μg) were subject to SDS-PAGE, followed by immunoblotting for IκBα.

inhibitors are nonspecific and therefore potentially very toxic. However, the IκBα "super-repressor," p6R-IκB$_{S32A + S36A}$, has also proven to be very effective in inhibiting activation of NF-κB[20]. Its ability to be genetically engineered with a PSA promoter provides specificity for prostate cancer cells and therefore makes it an ideal candidate for hormone-refractory prostate cancer gene therapy.

We report an efficient transduction of exogenous "super-repressor," p6R-IκB$_{S32A + S36A}$, NF-κB into both TNF-α-insensitive PC-3 cells and TNF-α-sensitive LNCaP cells. This "super-repressor" blocked IκBα phosphorylation, NF-κB translocation and activation, IL-6 production, and induced apoptosis in transfected PC-3 and LNCaP cells exposed to TNF-α. Our data demonstrate that p6R-IκB$_{S32A + S36A}$ is a powerful tool that can sensitize TNF-α-insensitive prostate cancer cells to undergo apoptosis. Moreover, blockage of IL-6 production decreases cell proliferation of androgen-independent prostate cancer cells.

MATERIALS AND METHODS

Cell Cultures. Androgen-sensitive LNCaP and androgen-insensitive PC-3 cells (American Type Culture Collection, Rockville, MD) were maintained at 37°C in an atmosphere of 5% CO_2 in RPMI 1640 (Life Technologies, Grand Island, NY) containing 1% antibiotic-antimycotic (10,000 units/ml penicillin G, 10,000 μg/ml streptomycin sulfate, and 25 μg/ml amphotericin B; Life Technologies) and supplemented with 10% fetal bovine serum (Life Technologies).

Transfections. The IκBα$_{S32A/S36A}$ plasmid used in this study was described previously (21, 33). Transfections were conducted by using SuperFect (Qiagen, Valencia, CA). The procedure was followed according to the manufacturer's protocol. Briefly, cells were seeded overnight at 60% confluency. The DNA plasmid (10 μg/10-cm dish or 2 μg/6-cm dish) containing the p6R-IκB$_{S32A + S36A}$ "super-repressor" was mixed with 10 or 25 μl of SuperFect, respectively. In control experiments, the empty eukaryotic expression vector, p6R, was similarly introduced into cells. Cells were incubated with the DNA mixture for 2 h at 37°C. Equal amounts of additional fresh media were then added to the cells. The cultures were incubated at 37°C for 24 h and replaced with fresh media. Cells were then treated with TNF-α (10 ng/ml) for 24 h.

Cytosolic and Nuclear Extracts. Cell pellets were washed in PBS, pelleted again, resuspended in buffer A [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 5 mM DTT, 5 mM phenylmethylsulfonyl fluoride and protease inhibitors (50 μg/ml antipain, 2 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin)] and placed on ice for 10 min. The cells were then vortexed and centrifuged for 10 s. The supernatant was placed in a separate tube, and 10 mM EDTA, 120 mM KCl, and 20% glycerol were added. This mixture was designated cytosolic extracts and stored at −80°C. The pellets were resuspended in equal volumes of buffer C [10 mM HEPES (pH 7.9), 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.8 M KCl, and protease inhibitors] and incubated for 20 min on ice. Samples were centrifuged for 5 min at 10,000 × *g* at 4°C. Supernatants were designated nuclear extracts and were stored at −80°C.

Western Blot Analysis. Equal amounts of cytosolic extracts (50 μg) were analyzed by SDS-PAGE, followed by Western blotting using a polyclonal rabbit IκBα antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive IκBα was detected using the enhanced chemiluminescence (ECL) light detecting kit (Amersham).

EMSA. NF-κB oligonucleotide probe (Santa Cruz Biotechnology) was labeled with [γ^{32}P]ATP to 50,000 cpm/ng using polynucleotide kinase. Nuclear extracts (5 μg) were incubated with 1 μg of poly(deoxyinosinic-deoxycytidylic acid) a 20-μl volume of gel shift reaction buffer [10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM DTT, 1 mM EDTA, and 5% glycerol] and 0.5

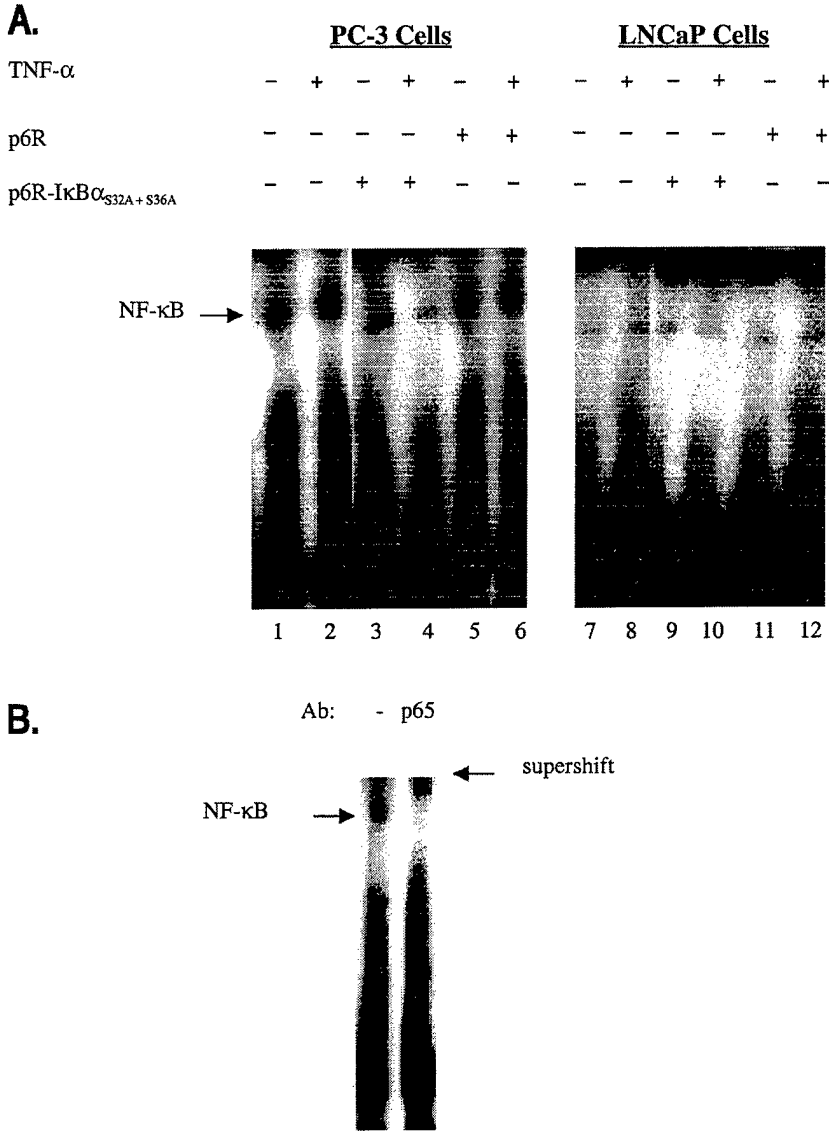

Fig. 4 The IκBα "super-repressor" blocks the stimulation of NF-κB by TNF-α in PC-3^T and LNCaP^T cells. *A*, PC-3 and LNCaP cells were first transfected with p6R-IκB$_{S32A + S36A}$ or the control p6R alone for 24 h before stimulation with 10 ng/ml of TNF-α for 24 h. Nuclear proteins (5 μg) were assayed for NF-κB DNA binding activity by EMSA using a radiolabeled consensus NF-κB site as a probe. *B*, nuclear protein (2 μg) was assayed for κB binding activity by EMSA. RelA antibody supershifting is indicated.

ng of labeled oligonucleotide probe for 20 min at room temperature. For supershifts with p65 antibody, nuclear extracts from untransfected PC-3 cells were preincubated with 1 μl of RelA antibody against the COOH-terminal portion of the molecule (Santa Cruz Biotechnology) for 15 min at room temperature before the addition of binding buffer and probe. DNA-protein complexes were resolved by electrophoresis through a 4% polyacrylamide gel containing 50 mM Tris (pH 7.5), 0.38 M glycine, and 2 mM EDTA. The gel was then dried and visualized by autoradiography.

RNA Extraction and Amplification. RNA was isolated from control, p6R, and mutant, p6R-IκB$_{S32A + S36A}$, transfected PC-3 cells using the TRIzol method (Life Technologies). Total RNA (10 μg) was amplified and measured using a mRNA IL-6 Quantikine ELISA (R&D Systems, Minneapolis, MN).

IL-6 ELISA. An IL-6 ELISA of cell culture supernatants from control, p6R, and mutant, p6R-IκB$_{S32A + S36A}$, transfected PC-3 cells was performed in triplicate according to the manufacturer's specifications (R&D Systems). Supernatants were removed at various time points after TNF-α (10 ng/ml) stimulation.

Apoptosis Detection. Annexin V fluorescent staining was assayed using an Annexin V apoptosis kit (Santa Cruz Biotechnology), and Apoptag fluorescent staining was detected using an immunohistochemistry kit (Intergen, Purchase, NY), according to manufacturer's protocol.

Statistical Analysis. Data are expressed as a means ± SE. Statistical significance was performed by the two-tailed Student *t* test for paired data and considered significant if *P*s were <0.05.

RESULTS

Blocked Degradation of IκBα in Transfected LNCaP (LNCaP^T) and PC-3 (PC-3^T) Cells. To inhibit TNF-α induced IκBα degradation, we transfected LNCaP and PC-3 cells

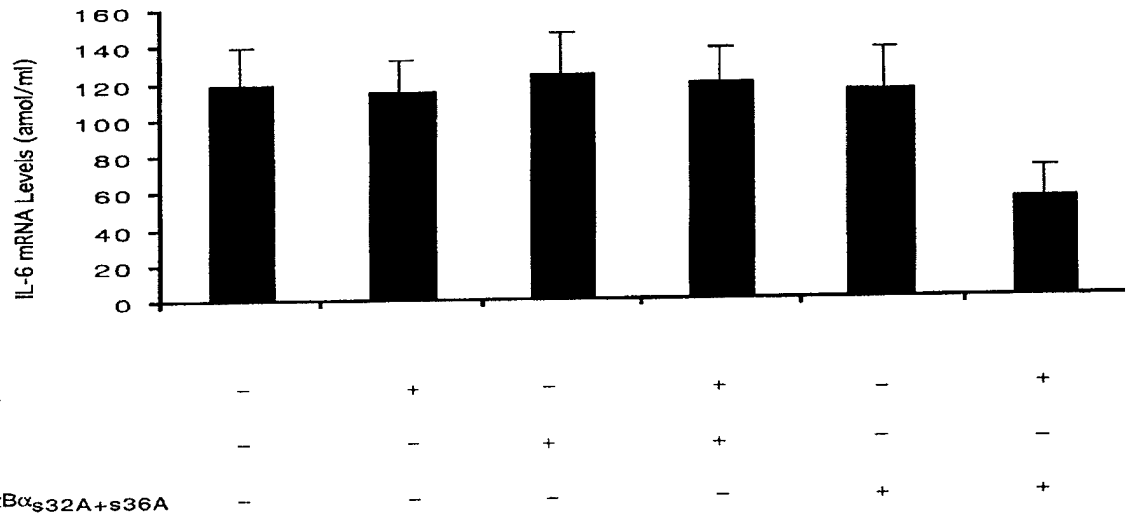

Fig. 5 The IκBα "super-repressor" blocks the stimulation of IL-6 mRNA production by TNF-α in PC-3^T cells. PC-3 cells were first transfected with p6R-IκB$_{S32A + S36A}$ or the control p6R alone for 24 h before stimulation with 10 ng/ml of TNF-α for 24 h. IL-6 mRNA was measured in total RNA with a Quantikine ELISA. Data represent the means of three independent samples; *bars,* SD.

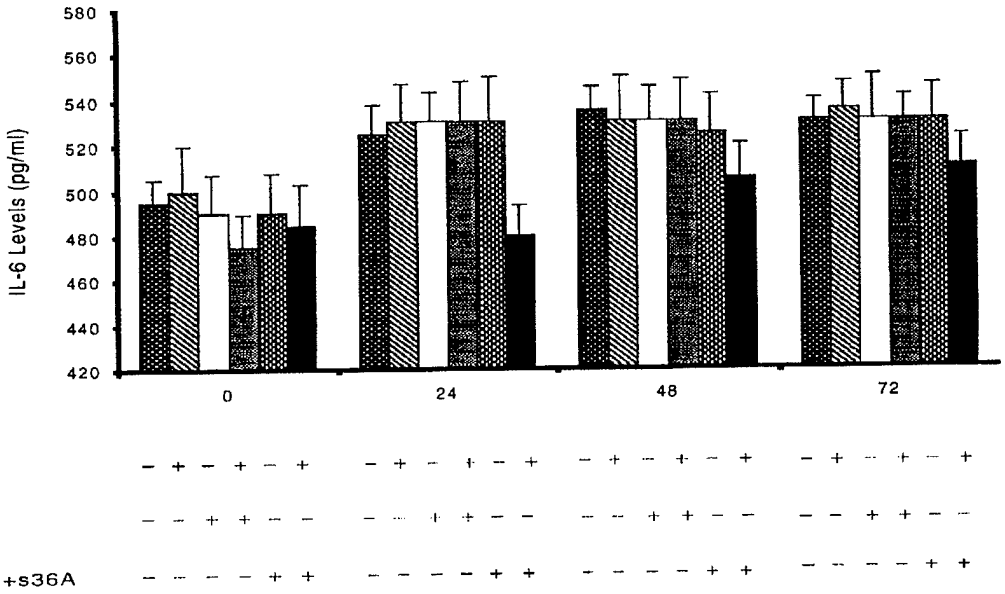

Fig. 6 The IκBα "super-repressor" blocks the stimulation of IL-6 secretion by TNF-α in PC-3^T cells. PC-3 cells were first transfected with p6R-IκB$_{S32A + S36A}$ or the control p6R alone for 24 h before stimulation with 10 ng/ml of TNF-α for 24 h. IL-6 was measured in the media by ELISA. Data represent the means of three independent samples; *bars,* SD.

with the IκBα "super-repressor," p6R-IκB$_{S32A + S36A}$, which is not susceptible to phosphorylation at NH_2-terminal serines 32 and 36 and is therefore resistant to subsequent degradation. Western blot analysis of cells transfected with p6R-IκB$_{S32A + S36A}$ and treated with 10 ng/ml of TNF-α for 24 h demonstrated blockage of IκBα phosphorylation and degradation (Fig. 3). The TNF-α-insensitive PC-3 cells constitutively degraded IκBα until its phosphorylation was blocked (Fig. 3, *Lane 4*). This phenomenon was present 24 h after TNF-α was added to the cultures. After 48 h in culture, p6R-IκB$_{S32A + S36A}$ alone could stop degradation of IκBα (data not shown). On the basis of these data, it appears that TNF-α expedites the blocked degradation of IκBα. The TNF-α-sensitive LNCaP cells only degraded IκBα in the presence of 10 ng/ml TNF-α (Fig. 3, *Lanes 8* and *12*), except where phosphorylation was blocked by the IκBα "super-repressor" (Fig. 3, *Lane 10*).

Inhibition of NF-κB Translocation and Activation. Nuclear extracts prepared from LNCaP^T and PC-3^T cells were analyzed by EMSA for their NF-κB DNA binding activity using a radiolabeled binding site as a κB probe. TNF-α strongly induced NF-κB activity in untransfected and control cells (Fig. 4*A*). Transfection with p6R-IκB$_{S32A + S36A}$ and stimulation with TNF-α (10 ng/ml) for 24 h significantly

reduced the cytokine-mediated NF-κB nuclear translocation (Fig. 4*A*, *Lanes 4* and *10*) In PC-3 cells without the IκBα "super-repressor" and TNF-α stimulation, the constitutive degradation of IκBα causes NF-κB to be constitutively expressed in the nucleus, where it transcribes antiapoptotic genes that cause TNF-α insensitivity. However, LNCaP cells only degrade IκBα in the presence of TNF-α (Fig. 4*A*, *Lanes 8* and *12*), except where the "super-repressor" is present (Fig. 4, *Lane 10*), and therefore have significantly less NF-κB in the nucleus. Again, after 48 h in culture p6R-$I\kappa B_{S32A + S36A}$ alone could decrease NF-κB translocation to the nucleus (data not shown). A supershift assay, with lysate obtained from untransfected PC-3 cells (Fig. 4*B*), was performed with a p65 antibody to demonstrate specificity to the NF-κB complex p65. Both a negative control without nuclear extracts as well as a competitive control using a 100-fold excess of unlabeled consensus mutant NF-κB were used to establish specificity of the reaction (data not shown).

Inhibition of *IL-6* Gene Expression by p6R-$I\kappa B_{S32A + S36A}$. The strong inhibition of NF-κB activity by p6R-$I\kappa B_{S32A + S36A}$ in prostate cancer cells suggested that TNF-α-mediated, NF-κB-dependent gene induction could be down-regulated by this reagent. As shown in Fig. 5, TNF-α induction of IL-6 mRNA expression is inhibited in p6R-$I\kappa B_{S32A + S36A}$ transfected PC-3 cells, when compared with untransfected and control cells, reaching a maximum of only 60 amol/ml.

We also investigated whether p6R-$I\kappa B_{S32A + S36A}$ would be able to prevent IL-6 secretion resulting from TNF-α and other stimuli. Fig. 6 demonstrates that PC-3 cells transfected with p6R-$I\kappa B_{S32A + S36A}$ and treated with TNF-α (10 ng/ml) have significantly decreased levels of IL-6 secretion when compared with untransfected and control cells. This is particularly apparent at 24 h after TNF-α stimulation, where IL-6 levels are decreased to 480 (pg/ml).

Mutant IκBα Significantly Sensitizes Prostate Cancer Cells to Apoptosis Induced by TNF-α. Sensitization of PC-3^T and $LNCaP^T$ cells with TNF-α (10 ng/ml) for 24 h induces apoptosis as assessed by morphological examination, propidium iodide staining, Annexin V staining, and Apoptag staining. Annexin V staining of cells transfected with p6R-$I\kappa B_{S32A + S36A}$ and sensitized with TNF-α demonstrate an increase in apoptotic activity (Fig. 7*A*) when compared with untransfected and control cells (Fig. 7, *B* and 7*C*). Fig. 8 shows columns illustrating the intensity of Apoptag staining in PC-3 and LNCaP cells, respectively. Again, when cells are transfected with the IκBα mutant and sensitized with TNF-α, there is a significant increase in apoptotic cell death.

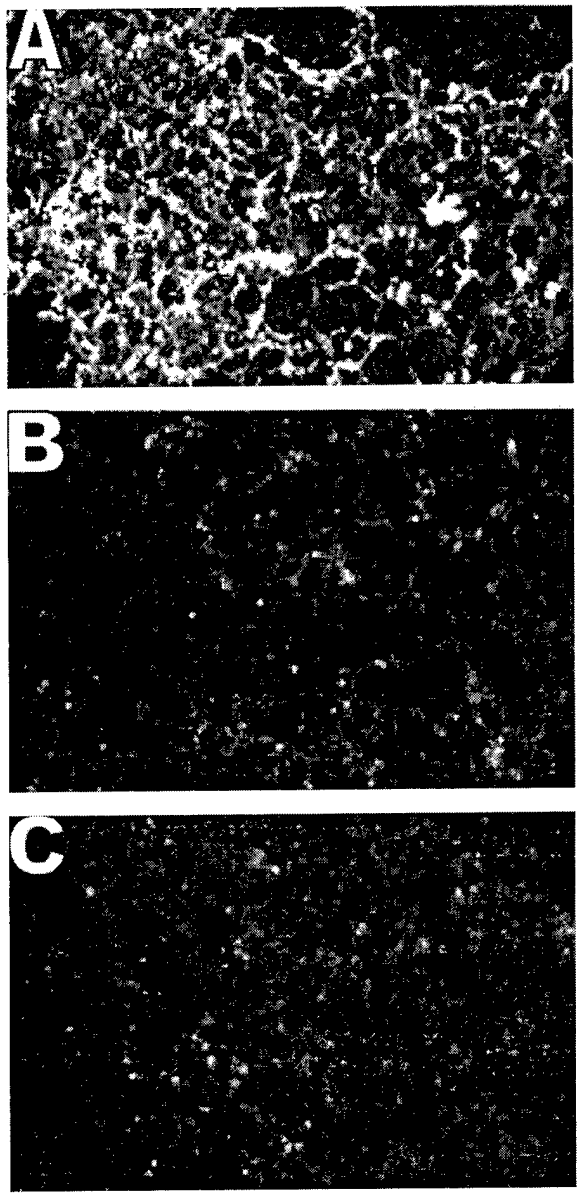

Fig. 7 The effect of NF-κB inhibition by p6R-$I\kappa B_{S32A + S36A}$ on TNF-α-induced apoptosis. Annexin V staining of apoptotic PC-3 cells after transfection with p6R-$I\kappa B_{S32A + S36A}$ and stimulation with TNF-α (10 ng/ml; *A*). Note the intense fluorescence of these cells. Stimulated LNCaP cells all experienced apoptosis (data not shown). Untransfected (*B*) and control (*C*) PC-3 cells with the same stimulation experienced only minimal apoptosis, and unstimulated PC-3 and LNCaP cells did not experience any apoptosis.

DISCUSSION

Prostate cancer patients experiencing a relapse in disease often acquire increased serum TNF-α levels when compared with untreated patients or patients in remission (9). These patients also experience a higher early mortality rate, and it seems likely that serum TNF-α levels increase in prostate cancer patients with end-stage disease (9). The prostate cancer cells found in relapsing patients are primarily androgen insensitive because of prior androgen ablation therapy. Studies have shown that these androgen-insensitive prostate cancer cells are also TNF-α insensitive (13). We proposed that this was attributable to NF-κB activation of antiapoptotic genes, such as those that cause TNF-α insensitivity. There are some cancer cells that are particularly sensitive to the cytotoxic effect of TNF-α (8). However, TNF-α has also been implicated in tissue remodeling and proliferation in some cell types (8). This paradox is because of two separate and distinct TNF-α-mediated pathways present in cells. One

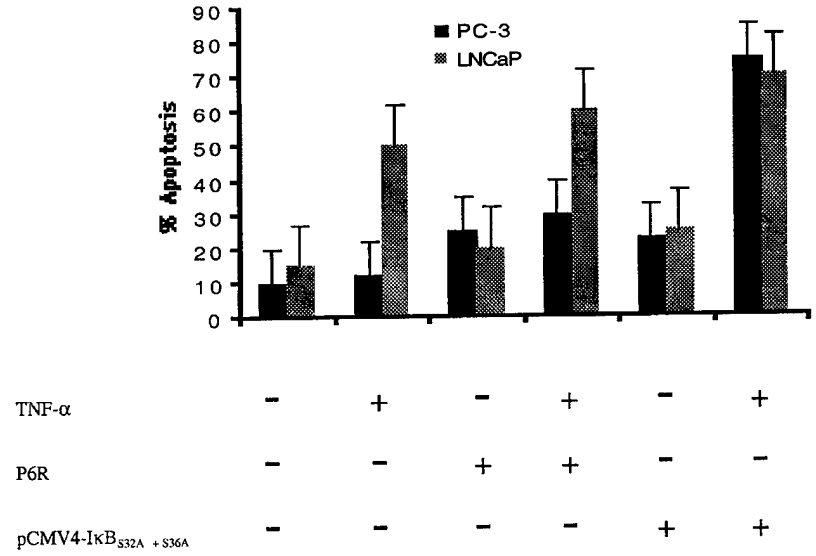

Fig. 8 The effect of NF-κB inhibition by p6R-IκB$_{S32A + S36A}$ on TNF-α-induced apoptosis. Apoptosis was examined by Apoptag fluorescence staining of PC-3 and LNCaP cells. The number of apoptotic and healthy cells was counted by a fluorescent microscope in randomly selected three fields/well, and the data are presented as a percentage of cells appearing apoptotic. Triplicate wells in each experiment were examined (means; *bars,* SE; $n = 4$).

pathway leads to apoptosis and the other to activation of protective antiapoptotic genes through NF-κB. Which pathway is activated depends on the TNF-α threshold of that particular cell. Typically, lower or normal levels of TNF-α induce the NF-κB protective pathway to keep the cell from undergoing unnecessary apoptosis, where higher or toxic levels induce apoptosis. TNF-α-insensitive cells, such as androgen-insensitive prostate cancer cells, do not experience TNF-α-induced apoptosis, even at the LD_{10} dose of 10 ng/ml. This is attributable to the constitutive degradation of IκBα and therefore NF-κB activation of antiapoptotic genes. Even TNF-α levels of 100 ng/ml could not force the TNF-α-insensitive cells into apoptosis. The fact that the androgen-insensitive prostate cancer cells were also TNF-α insensitive, coupled with the increased serum TNF-α levels of relapsing prostate cancer patients, lead us to believe that these cells were thriving in a toxic TNF-α environment attributable to the induction of NF-κB antiapoptotic genes.

This study provides important new insights into the effects of inhibiting NF-κB in TNF-α-insensitive prostate cancer cells. A wide range of intracellular components are implicated in TNF-α-induced cell killing, including pertussis toxin-sensitive guanine nucleotide binding protein (34), phospholipase A_2 (35), phospholipase D activation (36), and DNA damage (37). Despite which receptor sets off the cytotoxic effect, the various intracellular signals activated by TNF-α lead to activation of NF-κB (4). Prostate cancer cells up-regulate multiple NF-κB-responsive genes in response to TNF-α stimulation. These molecules may be involved in cell proliferation and metastasis, where modulation of their expression could be clinically beneficial. We have targeted our intervention at the IκB/NF-κB pathway because most of these genes are predominately regulated at the level of transcription.

After TNF-α stimulation, NF-κB maintains a balance between an inactive and active state that relies mostly on IκBα (21). NF-κB can be shifted to an inactive or active state by overexpression or degradation of IκBα (21). Previous approaches to inhibit NF-κB activity have focused on endothelial or mononuclear hemopoietic cells (21). Most strategies target IκBα through proteosome blockades, phosphorylation inhibition, and protein overexpression (38, 39). The method we used to block the NF-κB pathway in TNF-α-insensitive prostate cancer cells was transfection with an IκBα "super-repressor" that resists TNF-α induced phosphorylation and degradation. The IκBα protein used was a S32A/S36A mutant form of IκBα that mutated the inducible amino acid phosphoreceptor and therefore abolished the degradation process (21). Our approach was successful in maintaining IκBα levels, which selectively inhibited NF-κB p65 subunit nuclear translocation, NF-κB DNA binding activity, down-regulated the induction of NF-κB responsive gene *IL-6,* and induced apoptosis.

Androgen-independent prostate cancer cells spontaneously release high levels of IL-6 into the cell supernatant without exogenous stimulation (13). IL-6 is a cytokine with pleiotropic activities and has been shown to play a central role in immune host-defense mechanisms (40). This cytokine has been implicated in growth differentiation, inhibition, and proliferation, depending upon the nature of the responsive target cells (41). IL-6 has been shown to promote cell proliferation in androgen-independent prostate cancer cells, PC-3 (42). When these cells were transfected with the p6R-IκB$_{S32A + S36A}$ "super-repressor" and stimulated with TNF-α, IL-6 secretion and IL-6 mRNA production was decreased. Although our results demonstrate only partial inhibition of IL-6 production, we believe that this IL-6 is residual, because of the fact that the "super-repressor" only inhibits new gene transcription, and that we have demonstrated that p6R-IκB$_{S32A + S36A}$ significantly suppresses new IL-6 production by TNF-α-stimulated PC-3 cells.

Transfected prostate cancer cells stimulated with TNF-α were induced to proceed through apoptosis. With the NF-κB pathway blocked by the IκBα "super-repressor," the cells were forced to proceed though TNF-α-induced apoptosis. Our results indicate that the previously TNF-α-insensitive prostate cancer

cells were made sensitive because of transfection with p6R-$IκB_{S32A + S36A}$. Once made sensitive, the cells experienced apoptotic cell death upon stimulation with TNF-α.

The complex signal transduction pathway, beginning from the binding of a cytokine to its receptor and leading to NF-κB transcriptional activity, provides many opportunities for therapeutic intervention (20). Other studies have demonstrated that p6R-$IκB_{S32A + S36A}$ can be successfully incorporated within the recombinant replication-deficient adenovirus, giving rise to possible clinical use (20, 21). In future studies, we propose using a PSA-promoter, pPSA-630 (43), added to p6R-$IκB_{S32A + S36A}$, that would specifically target only PSA-secreting cells such as those found in prostate cancer relapsed patients. This PSA promoter can only be activated in the presence of specific transcription factors present only in PSA-producing cells. One such transcription factor is the androgen receptor present in prostate cancer cells. This gene therapy might be beneficial to advanced prostate cancer patients and would avoid the severe toxicities associated with other IκB inhibitors, such as ALLN and MG-132, by specifically targeting prostate cancer cells. With NF-κB activation blocked, prostate cancer cells could proceed through apoptosis using the elevated serum TNF-α levels already present in patients with relapsed prostate cancer.

In conclusion, this study extends known antiapoptotic roles of NF-κB to prostate cancer cells and emphasizes that the blockage of NF-κB can selectively sensitize previously insensitive cells to apoptosis by TNF-α. These findings suggest a potential new therapeutic tool for prostate cancer gene therapy.

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The Study of Gemcitabine in Combination with Other Chemotherapeutic Agents as an Effective Treatment for Prostate Cancer

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Abstract. *Background: Gemcitabine has demonstrated clinical activity against several common cancers. Our studies examine the ability of gemcitabine, both alone and in combination with other chemotherapeutic agents, to inhibit the in vitro and in vivo growth of several prostate cancer cell lines. Materials and Methods: Cultures of LNCaP, PC-3 or MLL cells were exposed to either gemcitabine or other appropriate agents for specified amounts of time. Cells were lysed and nuclei counted utilizing a Coulter Counter. For in vivo experiments, animals were injected with 1 x 10^5 MLL cells subcutaneously into the right flank. Animals were treated as indicated for 14 days. Tumors were then excised, weighed and measured. Results: In both human (PC-3 and LNCaP) and rat prostate (MLL) cancer cell lines our studies demonstrated gemcitabine had a strong effect in vitro, with an IC_{50} of approximately 500 nM in the human lines and 10 nM in MLL cells. In vivo , studies using the Dunning prostate cancer model in Copenhagen rats resulted in a dose response inhibition of tumor growth, with an 80% decrease in tumor size in rats treated with gemcitabine at 10 mg/kg. Conclusions: Our results demonstrated the potent activity of gemcitabine against prostate cancer in the Dunning rat model and suggest the addition of paclitaxel may not aid in this activity.*

Prostate cancer is the most frequently diagnosed cancer and the second leading cause of cancer death in men in the United States. Projected estimates for 1999 indicate that there will be approximately 179,300 newly diagnosed prostate cancers and 37,000 men will die from this disease (1). Although organ confined prostate cancer is potentially curable with radical prostatectomy and/or radiation, treatment of locally advanced or metastatic disease still remains palliative (2). A fundamental key to better treatments is the development of improved chemotherapy regiments that have a better efficacy and a lower toxicity. While many single-agent and multi-drug schedules have been helpful in the fight against prostate cancer, new combinations are continuously being sought to impede cancer spread and lower mortality rates.

Gemcitabine (2',2'-difluorodeoxycytidine, dFdC, Gemzar) is an S-phase-specific, fluorine-substituted pyrimidine which has demonstrated clinical activity against several common cancers. In previous studies, gemcitabine has been tested in the treatment of hematologic cancers and found to be active (3, 4). It was then found to be effective in solid tumors (5) and carried into clinical trials. Phase II and Phase III clinical trials utilizing gemcitabine have demonstrated significant activity in patients with advanced breast (6, 7), ovarian (8-11), non-small cell lung (12, 13), pancreatic (14, 15), head and neck (16) and bladder cancers (17-20). These studies also indicate that gemcitabine is well tolerated, with the most common dose-limiting toxicity being myelosuppression. Other side effects include flu-like symptoms including low-grade fever, fatigue and malaise (21). Likewise, gemcitabine alone showed similar antitumor activity and a better safety profile when compared with a combination of cisplatin and etoposide in a phase II study of patients with non small-cell lung cancer (22).

The parental form of gemcitabine is inactive but is progressively phosphorylated intracellularly to the active gemcitabine diphosphate and gemcitabine triphosphate forms. Gemcitabine diphosphate inhibits ribonucleotide reductase (23) and the triphosphate form actively competes with dCTP24. Incorporation of gemcitabine triphosphate into DNA results in DNA chain termination (24). It has also been demonstrated that this metabolite can incorporate into RNA

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Key Words: Gemcitabine, paclitaxel, combination chemotherapy, Dunning rat model.

thus potentially inhibiting or altering RNA synthesis (25).

It has been the philosophy of many clinicians that although single agents are useful in the treatment of cancers, combinations of agents with different mechanisms of actions may be the best treatment. Because of the ability of gemcitabine to act at different stages of cell division, it appears to be a reasonable choice to study it in combination with other agents (26). There have been several reports which support this hypothesis. Gemcitabine in combination with cisplatin has been shown to have either an additive or a synergistic effect *in vitro* and *in vivo* in a number of different tumor systems (27-30). Similarly, treatment with gemcitabine with vindesine was shown to have a synergistic cytotoxic effect when used against a lung cancer cell line (31). Clinical trials of gemcitabine with carboplatin and paclitaxel have shown promising results in patients with non-small cell lung cancer (32). Phase I trials are also being conducted with gemcitabine and etoposide producing positive results (33).

The majority of published work on gemcitabine has not addressed the use of gemcitabine, either as a single agent or in combination with other chemotherapeutic agents to treat prostate cancer. A study by Cronauer *et al* examine gemcitabine as a single agent in the *in vitro* treatment of the PC-3, DU-145 and LNCaP human prostate cancer cell lines (34). Their results demonstrated gemcitabine had a strong effect on the prostate cancer cell lines, but did not effect normal epithelial cell growth (34). Our laboratory has previously demonstrated the synergistic activity of several chemotherapeutic combinations for the treatment of hormone refractory prostate cancer both in preclinical studies and in clinical trials (35-39). In this study, we report the *in vitro* and *in vivo* results of our study of the combination of various chemotherapeutic agents with gemcitabine using several prostate cancer cell lines.

Materials and Methods

Materials. Gemcitabine HCl was acquired from Eli Lilly and Co. (Indianapolis, IN) as an injectable form containing minimal amounts of mannitol and sodium acetate. Solutions of gemcitabine were made fresh daily at various concentrations using sterile deionized water. Injectable taxol (paclitaxel) suspended in Cremphor EIR and dehydrated alcohol was obtained from Bristol-Myers Squibb Co (Princeton, NJ). Taxol was diluted for experiments using sterile deionized water. Paraplatin (Carboplatin: cis-diammine [1,1-cyclobutane-dicarboxylato]platinum) as an injectable form was obtained from Bristol-Meyers-Squibb Co. Carboplatin was reconstituted in sterile water. Etoposide was purchased from Sigma Chemical Co (St. Louis, MO) in a dry powder form and was reconstituted in dimethylsulfoxide (DMSO). Estramustine was obtained from Pharmacia (Sweden) and was reconstituted in sterile water.

Cell culture. The MAT-Ly-Lu (MLL) subline of the Dunning R-3327 prostate adenocarcinoma line was a gift from Dr. John Isaacs (Johns Hopkins University, Baltimore, MD). The human prostate cancer cell lines PC-3 and LNCaP were obtained from American Type Culture Collection (Rockville, MD). All lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin.

Animals. Male Copenhagen rats were obtained from Harlan Sprague-Dawley (Indianapolis, IN). The animals averaged 270g each and were fed a diet of standard rat chow and water ad libidum. Carbon dioxide was used as a euthanasia method at the termination of experiments.

In vitro cell viability assays. For cytoxicity assay, triplicate T-25 flasks were seeded in the above media with initial densities of between 5×10^4 -2×10^5 cells per T-25 depending on cell line (either LNCaP, PC-3 or MLL). The cells were allowed to grow in normal growth media for 48 hours. Fresh media was then added to cultures containing the appropriate agent at varying concentrations (0.1 nM to 1000 nM). An appropriate vehicle was added to control flasks. Cells were allowed to grow for either 48 or 96 hours depending on experimental conditions. Media was changed every 48 hours.

Growth of cultures was monitored using the procedures described by Weise *et al* (40). Briefly, cells were allowed to swell in a hypotonic HEPES buffer (2 ml/T25; 0.01 M HEPES, 0.015 M magnesium chloride) for 10 minutes at room temperature. The cells were then incubated with detergent [200 µl/T25 of 0.13 ethylhexadecyldimethylammonium bromide (Eastman Kodak, Rochester, NY) in 3% v/v of acetic acid (Fisher, Pittsburgh, PA)] for 10 minutes with agitation to lyse the cells. The resulting nuclei suspension was diluted to appropriate volumes with filtered saline and counted with a Coulter Counter (41).

In vivo studies. Utilizing an Animal Investigation Committee approved protocol, male Copenhagen rats were injected with 1×10^5 MLL cells subcutaneously into the right flank on day 0. Rats were randomized into groups of 10 on day 4 after injections. Animals were given intraperitoneal (IP) injections of gemcitabine at indicated doses on days 4 and 9 after implantation of tumor cells. Animal treated with taxol were also given IP injections on appropriate days at a concentration of 2 mg/kg. Animals were then sacrificed on day 14, the tumors were then excised and measured. For the regression study, animals were injected as above on day 0. The rats were monitored until a 1 cm^2 tumor was present on the right flank, at approximately day 12. Except for controls, rats were treated by IP injection of gemcitabine. The injections were repeated on day 16 and the animals sacrificed on day 20. The tumors were then excised and measured.

Results

In Vitro Toxicity Studies. Treatment of the human prostate cancer cell lines LNCaP and PC-3 with gemcitabine alone demonstrated a moderate cytotoxic effect, with an IC_{50} (defined as the concentration of agent which yields 50% viability of cells when compared to control cell counts) of approximately 500 nM for each cell line (Figure 1A). Increasing concentrations to 1000 nM had minimal effect (Figure 1A). In contrast, gemcitabine proved to have a strong cytotoxic effect on MLL cells, with an IC_{50} of approximately 10 nM (Figure 1B). Maximum cytotoxic effect was observed at 100 nM with a roughly 90% growth inhibition observed (Figure 1B). Higher concentrations of gemcitabine proved less effective (data not shown).

The combination of gemcitabine with other chemotherapeutic agents was also tested *in vitro* using LNCaP cultures (Table I). Control experiments were performed with each agent alone in culture (Figure 2; data not shown) to determine appropriate concentration of agent to use in the combination studies with gemcitabine. Carboplatin (200 nM) when used alone did not inhibit cell growth (Table I). In

Table I. *Evaluation of Gemcitabine in combination with other chemotherapeutic agents in the growth of LnCaP cultures*[1].

Agent[2]	Viability (%) with Gem.[2], 96 hr.	Viability (%) with Agent, 96 hr.	Viability (%) with Gem. + Agent, 96 hr.	Viability (%) with Gem., 48 hr.; Agent, 48 hr.[3]	Viability (%) with Agent, 48 hr.; Gem. 48 hr.[3]
Carboplatin	84 ± 4.9%	101 ± 5.1%	100 ± 5.8%	106 ± 4.5%	109 ± 6.9%
Estamustine	84 ± 4.9%	107 ± 3.7%	80 ± 7.3%	95 ± 1.7%	90 ± 3%
Etoposide	84 ± 4.9%	82 ± 6.3%	74 ± 2.8%	82 ± 7.1%	97 ± 5.1%
Paclitaxel	82.7 ± 3.8%	48.4 ± 3.4%	28.5 ± 2.5%	62.3 ± 13.2%	62.5 ± 2.5%

[1]Each compound was evaluated as a single agent for its effect on the growth of LnCaP cultures. Concentrations ranges of 0.1-100,000 nM were examined. For these studies, concentrations were chosen based on the following criteria: 1) Previous results demonstrating an additive or synergistic response of cytotoxic at this concentrations 2). Physiologicial relevance.

[2]The following concentrations were used: Carboplatin, 200 nM; Estramustinte, 100 nM; Etoposide, 50 nM; Gemcitabine (Gem), 100 nM; and Paclitaxel, 1 nM.

[3]For multi-agent incubations, cells were first incubated with first agent for 48 hr. Flasks were then rinsed with HBSS and second agent was added for 48 hr.

combination with gemcitabine (100 nM) no growth inhibition of LNCaP cultures was observed at any of the combination sequence examined (Table I). This effect was also observed in PC-3 cultures (data not shown). Estramustine (100 nM) also had no effect on LNCaP cultures when used alone (Table I). In combination with gemcitabine, similar growth inhibition was observed as when gemcitabine was used alone (Table I). Furthermore, when these agents were given sequentially in culture, rather than together, the combination of estramustine and gemcitabine did not show a greater cytotoxic effect than gemcitabine alone (Table I). Etoposide (50 nM) alone showed minimal cytotoxicity in LNCaP cultures, with roughly an 18% growth inhibition (Table I). In combination with gemcitabine, etoposide demonstrated a slightly more toxic profile than when either was used alone (Table I). Sequential addition of agents again showed little difference (Table I). Similar results were observed in PC-3 cultures (data not shown).

Treatment of LNCaP cultures with the combination of paclitaxel and gemcitabine was also examined. Paclitaxel alone proved to be quite effective in inhibiting growth of all cultures examined (Figure 2) (42). When LNCaP cultures were exposed to both agents in tandem, an additive growth inhibition was observed (Table I; Figure 3). While treatment with gemcitabine and paclitaxel as single agents resulted in an 82% and 48% growth inhibition, respectively; the combination of the two agents resulted in a 28% inhibition of growth (Table I; Figure 3). When gemcitabine and paclitaxel were added sequentially to growth media, the combination of the two resulted in a growth inhibition greater than gemcitabine alone. Sequential addition of the two agents did not prove to be better than the addition of paclitaxel alone, however. This additive effect of the gemcitabine and paclitaxel was observed in all three lines examined (Figure 3). Sequential addition, however, proved less effective that gemcitabine alone in PC-3 cultures (Figure 3). In MLL cells, the addition of gemcitabine first with subsequent addition of paclitaxel 48 hours later proved to be much more toxic to these cultures than the reverse sequential addition of paclitaxel and then gemcitabine (Figure 3).

In vivo toxicity studies. The ability for gemcitabine to inhibit tumor growth *in vivo* was examined in the Dunning prostate cancer model using Copenhagen rats. Treatment of animals with dosages of 1, 5 and 10 mg/kg of gemcitabine resulted in a dose response inhibition of tumor growth, with an 81% decrease in tumor size in the rats treated with gemcitabine at 10 mg/kg (Figure 4). The treated rats showed no significant decrease in body weight (average of 224 gms) when compared to the control group (average of 220 gms). No outward signs of toxicity were present (data not shown). Regression studies were performed in which tumors were allowed to develop to 1 cm^2 in size and then the animals were subsequently treated with gemcitabine. Results showed an overall decrease in tumor weight of 60% and 56% compared to the control group. The treated groups showed an average of a 12% decrease in body weight as compared to controls. No other physical evidence of toxicity was present.

Guided by the results of the *in vitro* experiments described above, tumor implanted animals were treated with the combination of gemcitabine and paclitaxel. The use of paclitaxel alone resulted in approximately 60% decrease in tumor size (Figure 5). Using gemcitabine alone, however, or in any combination with paclitaxel decreased tumor weight drastically (average of 90% growth inhibition) when compared to control animals (Figure 5). In vivo, the use of paclitaxel appeared to only slightly enhance the ability of gemcitabine in inhibiting tumor growth in animals (Figure 5). By the end of 14 days, however, the animals treated with the combination of paclitaxel and gemcitabine began to show signs of toxicity, with their coats displaying a lack of grooming and the development of dermatitis on their noses. Two animals treated with the drug combination were found deceased on the scheduled day of termination. On average, the animals

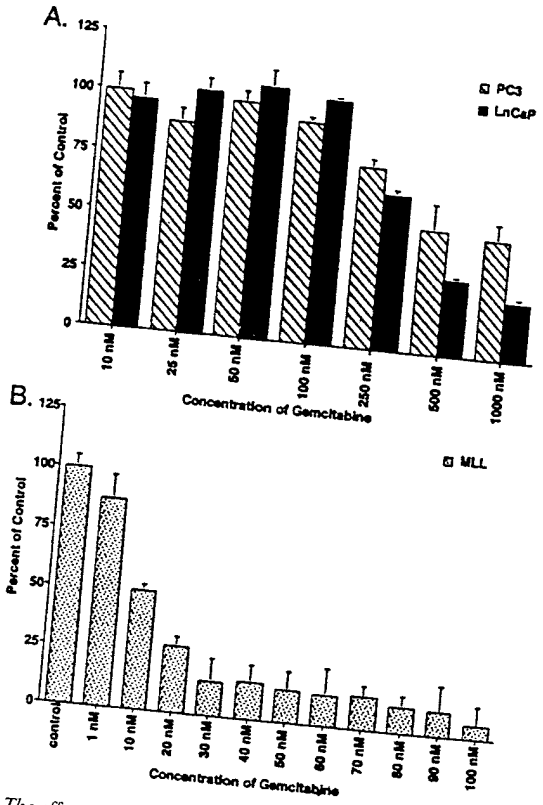

Figure 1. *The effect of gemcitabine on the in vitro growth of prostate cancer cell lines. Cultures were plated in triplicate T25 flasks at either 5 x 10^4 (MLL) or 2 x 10^5 (PC3 and LnCaP) cells per flask. After 48 hr., fresh media was added containing gemcitabine at indicated concentrations. Cells were allowed to incubate in this media for 48 hrs. After 48 hrs., cells were lysed and nuclei counted as indicated in the Materials and Methods. Bars represent the average cell viability (n=3) as percent of control with percent relative error.*

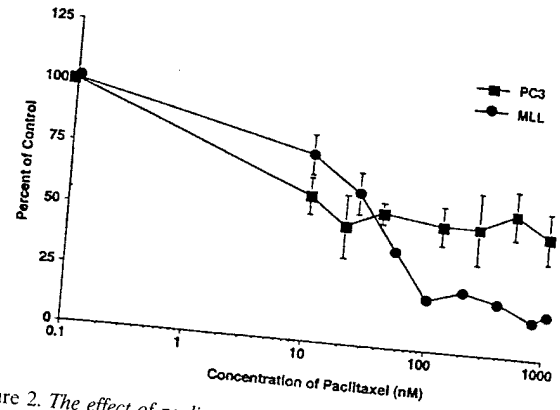

Figure 2. *The effect of paclitaxel on the in vitro growth of prostate cancer cell lines. Cultures were plated in triplicate T25 flasks at either 5 x 10^4 (MLL) or 2 x 10^5 (PC3) cells per flask. After 48 hrs, fresh media was added containing paclitaxel at indicated concentrations. Cells were allowed to incubate in this media for 48 hrs. After 48 hrs., cells were lysed and nuclei counted as indicated in the Materials and Methods. Bars represent the average cell viability (n=3) as percent of control with percent relative error.*

treated with the combination of drugs lost 16% of their body weight compared to the controls.

Discussion

The pyrimidine nucleotide derivative gemcitabine has been used in the treatment of a number of cancers, including pancreatic, ovarian , non-small cell lung, pancreatic, head and neck and bladder (6-20). The studies presented here and others demonstrate the usefulness of this drug in the treatment of prostate cancer. Used as a single agent, both *in vitro* and *in vivo*, gemcitabine showed significant cytotoxic effects. This study supports the original study by Cronauer *et al* (34) who demonstrated a strong cytotoxic activity of gemcitabine against several prostate cancer cell lines *in vitro* (IC_{50} obtained was much higher in our study for the human prostate cancer cell lines examined (PC-3 and LNCaP). This is possibly due to different experimental procedures. To ensure the cultures were in log phase growth before treatment, we allowed cells to incubate in normal media for 48 hours. Earlier studies by our group demonstrated the ultra-sensitivity of these cultures to chemotherapeutic agents when not allowed to recover from tissue culture manipulation (i.e. trypsin treatment or cell scraping). Nonetheless, both studies indicated the cytotoxic activity of gemcitabine against prostate cancer cells.

Our study further examined the ability of gemcitabine to inhibit tumor growth *in vivo*. Tumor sizes were greatly reduced in animals treated with all concentrations examined. Likewise, gemcitabine showed a cytotoxic effect against tumors grown to a size of 1 cm^2 before treatment began, indicating the usefulness of gemcitabine with established tumors. This again supports data in other tumor model systems (34,43,44). Moreover, this drug appears to be well tolerated in the animals with minimal toxicities observed.

In combination with other chemotherapeutic agents, gemcitabine along with paclitaxel gave the most promising results *in vitro*. Unlike other studies in different tumor systems (30, 32, 46), we did not observe a synergistic response between a platinum derivative and gemcitabine. These studies, however, examined the effect of cisplatin with gemcitabine, whereas our studies examined the effect of carboplatin. In our studies, carboplatin has not proven to be effective in inhibiting the *in vitro* growth of LNCaP or PC-3 cells (data not shown). *In vitro* assays examining the combination of gemcitabine and paclitaxel on these cultures, however, demonstrated additive results. It should be noted that each cell line exhibited different sensitivities to paclitaxel, with LNCaP cultures being the most sensitive (IC_{50} of approximately 8nM) and MLL cultures being the least sensitive (IC_{50} of approximately 50 nM). When furthering our studies *in vivo* , however, this additive effect was not observed. Although it appeared paclitaxel might have some effect when combined with gemcitabine, this effect was masked by the

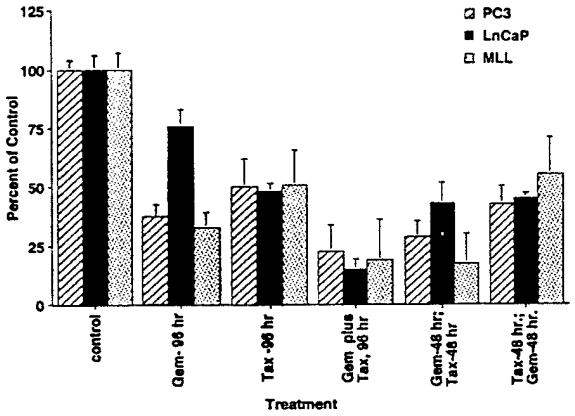

Figure 3. *The effect of gemcitabine and paclitaxel on the in vitro growth of prostate cancer cell lines. Cultures were plated in triplicate T25 flasks at either 5 x 10^4 (MLL) or 2 x 10^5 (PC3 and LnCaP) cells per flask. After 48 hr., fresh media was added containing gemcitabine (Gem) or paclitaxel (Tax), either alone or in combination, at the following concentrations: PC-3- 100 nM gemcitabine, 40 nM paclitaxel; LnCaP- 200 nM gemcitabine, 1 nM paclitaxel; MLL- 2.5 nM gemcitabine, 20 nM paclitaxel. Cells were allowed to incubate in this media for 48 hrs. Cultures were then rinsed with HBSS and fresh media containing the appropriate agent added. After 96 hrs., cells were lysed and nuclei counted as indicated in the Materials and Methods. Bars represent the average cell viability (n=3) as percent of control with percent relative error.*

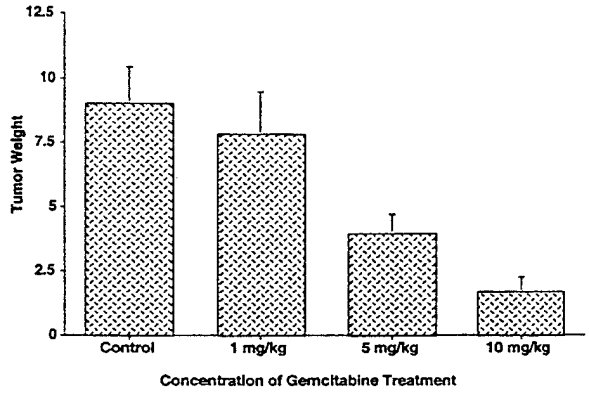

Figure 4. *The effect of gemcitabine on the in vivo growth of the MLL cell line in the Copenhagen rat. Animals were subcutaneously injected with 1 x 10^5 MLL cells in the right flank. Animals were treated by IP injection with indicated doses of gemcitabine on days 4 and 9 after tumor implantation. Animals were sacrificed 14 days after implantation, the tumors excised and measured. Bars represent the average tumor weight (n=10) with standard error.*

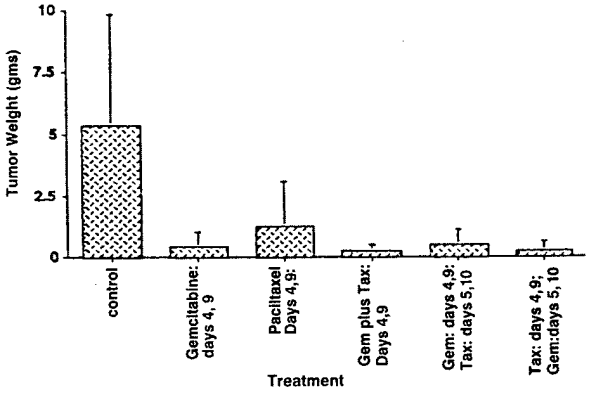

Figure 5. *The effect of gemcitabine and paclitaxel on the in vivo growth of the MLL cell line in the Copenhagen rat. Animals were subcutaneously injected with 1 x 10^5 MLL cells in the right flank. Animals were treated by IP injection with 5 mg/kg gemcitabine on days 4 and 9 after tumor implantation. Paclitaxel was administered by IP injection on days indicated at a concentration of 2 mg/kg. Animals were sacrificed 14 days after implantation, the tumors excised and measured. Bars represent the average tumor weight (n=10) with standard error.*

anti-tumor effect of gemcitabine alone in these animals. Gemcitabine has a strong effect in the inhibition of tumor growth, either with or without paclitaxel. The addition of paclitaxel did appear to decrease tumor size, although this was not statistically significant. Furthermore, this combined regiment appeared not well tolerated in these animals. Animals developed outward signs of toxicity with hair and weight loss. This toxicity, however, appears not to be relevant in a clinical setting as patients appear to tolerate this regiment well in reported Phase I/II trials (32). It appears that gemcitabine is much more toxic in rats than observed in the clinical setting.

Although our data suggests that paclitaxel and gemcitabine may prove to be an effective combination in the treatment of prostate cancer, further studies are needed. Recently, there have been reports emphasizing the importance of scheduling and dosing when examining combination trials with gemcitibine (46). This may be the reason for the less than additive effect observed *in vivo*.

In conclusion, our data demonstrates the potent anti-tumor activity of gemcitabine in prostate cancer. It appears that while paclitaxel demonstrated an additive effect *in vitro*, the *in vivo* combination proved too toxic to the animals to gain a concrete effect. Perhaps further studies, examining the dosing and scheduling of these two agents may prove more informative. Preliminary experiments in which estramustine was added to these cultures along with gemcitabine and paclitaxel in tandem suggests that this may be an effective combination (M. Mahoney and K. Pienta, unpublished results). Further studies that examine the combination of gemcitabine with other chemotherapeutic agents are warranted.

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Treatment of Androgen-Independent Prostate Cancer Using Antimicrotubule Agents Docetaxel and Estramustine in Combination: An Experimental Study

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BACKGROUND. Estramustine in combination with other chemotherapeutic agents has demonstrated synergy in hormone-refractory prostate cancer. Docetaxel has demonstrated antineoplastic activity in a variety of chemotherapeutic-unresponsive tumors. We evaluated the effects of estramustine and docetaxel in preclinical models of prostate cancer.

METHODS. Cell viability of PC-3 and MAT-LyLu (MLL) cells were assessed 48 hr after drug treatment. For in vivo studies, each flank of five animals in six groups was injected with 1×10^6 MLL cells: control, estramustine, docetaxel (low- and high-dose), and low- and high-dose docetaxel with estramustine. Animals were treated on days 4 and 11, and sacrificed on day 14.

RESULTS. The IC_{50} value for docetaxel was 2 nM in the PC-3 cells and 40 nM in the MLL cells. The addition of 100 nM of estramustine did not alter the IC_{50} value for PC-3 cells. In the MLL cells, however, the IC_{50} value was lowered to 15 nM. In vivo, low-dose docetaxel with estramustine demonstrated antineoplastic activity similar to that of high-dose docetaxel alone, suggesting additive activity between the drugs.

CONCLUSIONS. These results demonstrate that when used in combination, docetaxel and estramustine can be more effective at lower dosages than when the individual drugs are used alone. *Prostate 44:275–278, 2000.* © 2000 Wiley-Liss, Inc.

KEY WORDS: **docetaxel; estramustine; MLL; PC-3; prostate**

INTRODUCTION

Prostate cancer is the most common malignancy diagnosed in men, and the second leading cause of death in American males [1]. There are limited treatment options, and prognosis continues to be dismal in metastatic hormone-refractory prostate disease. In newly diagnosed metastatic disease, hormone therapy controls symptoms in 80–90% of patients . The median duration of response is approximately 2–3 years. Chemotherapy is the next option; however, the response induced by chemotherapy is generally limited and of short duration, with an overall objective response of 8.7% for single agents and approximately 50% for newer regimens. Cancer patients are often debilitated or immunocompromised and, therefore, chemotherapy is not always feasible or well-tolerated [2]. Treatment of metastatic disease is considered palliative, since no chemotherapeutic regimen has shown a survival benefit in a randomized clinical trial. Due to these limitations, new agents and strategies clearly needed.

Estramustine phosphate (Emcyt, Estracyt), a nonnitrogen mustard carbamate, binds to microtubule-associated proteins, inhibits assembly, and disrupts

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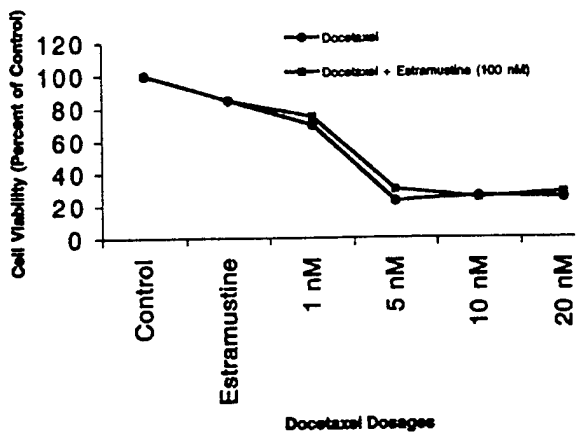

Fig. 1. In vitro analysis of PC-3 cells treated with Taxotere demonstrated an IC_{50} value of approximately 2 nM. The addition of Emcyt (100 nM) did not significantly alter this IC_{50} value.

microtubule organization in vitro [3]. It is this cellular mechanism, and not the hormonal effect associated with the estrogen moiety or alkylating activity due to nitrogen mustard, that is responsible for the cytotoxic effects of estramustine in hormone-refractory prostate cancer [3]. Estramustine has also been shown to reduce the level of serum prostate-specific antigen (PSA) [4].

Docetaxel (Taxotere), a member of the taxane family, binds to tubulin, promoting microtubule assembly and microtubule bundling [5,6]. Like other members of the taxane family, docetaxel stabilizes spindle microtubules, impairing mitosis and blocking progression through the cell cycle [5]. It demonstrates significantly longer cellular affinity and uptake, and slower cellular efflux than paclitaxel, which prolongs the duration of cell drug exposure [7]. Docetaxel is also approximately twice as efficient as paclitaxel in stabilizing microtubules [8].

Estramustine in combination with other chemotherapeutic agents has demonstrated synergy in hormone-refractory prostate cancer. Docetaxel has demonstrated antineoplastic activity in a variety of previously chemotherapeutic-unresponsive tumors. We here evaluated the in vitro and in vivo effects of estramustine and docetaxel in preclinical models of prostate cancer.

MATERIALS AND METHODS

Drugs and Reagents

Estramustine phosphate (estradiol-3-N-bis-[2-chloroethy]-carbamate-17-beta-dihydrogen disodium phosphate) was purchased from Hoffmann-LaRoche (Nutley, NJ). Docetaxel (Taxotere) was purchased from Rhone-Poulenc Rorer (Collegeville, PA) as a prepared sterile stock solution of 10 mg/ml.

Cell Culture

The prostate adenocarcinoma cell line PC-3 (American Type Culture Collection, Rockville, MD) and the metastatic MAT-LyLu (MLL) subline of the Dunning R-3327 rat prostate adenocarcinoma line were grown and maintained at 37°C in an atmosphere of 5% CO_2 in RPMI-1640 medium (Life Technologies, Grand Island, NY) containing 1% antibiotic-antimycotic (penicillin G, 10,000 U/ml; streptomycin sulfate, 10,000 μg/ml; and amphotericin B, 25 μg/ml) (Life Technologies) and supplemented with 10% fetal bovine serum (Life Technologies). Stock drug was added to the cell culture medium to reach the reported concentrations.

Cell Growth and In Vitro Cytotoxicity Assay

Cell adhesion was used as a marker of cell viability. At hr 0, 2×10^5 PC-3 and MLL cells, per T25 flask, were plated in triplicate. At hr 48, increasing doses of docetaxel alone, as well as in combination with +100 nM estramustine, were added to the flasks. After an additional 48 hr of incubation, the cells were lysed and the nuclei counted (Z1 Coulter Counter, Coulter, Hialeah, FL).

Animals

Male Copenhagen rats were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Methoxyflurane (Pittman-Moore, Washington Crossing, NJ) was used as inhalation anesthetic for injections and surgical procedures.

In Vivo Animal Tumor Model

Animals, 5 per group, were injected with 1×10^6 MLL cells subcutaneously into each flank on day 0. There were 5 rats per treatment and control group, respectively.

Treatment groups consisted of the following: 1) control, 2) estramustine (7 mg/kg), 3) low-dose docetaxel (7 mg/kg), 4) high-dose docetaxel (11.6 mg/kg), 5) low-dose docetaxel and estramustine, and 6) high-dose docetaxel and estramustine.

On day 0, animals were injected with 1×10^6 MLL cells in each flank. On days 4 and 11, animals were given intraperitoneal injections of the drugs. On day 14, animals were sacrificed, the tumors were harvested, and the tumor weights were noted. Paired Student's *t*-test was used to compare control and treatment groups.

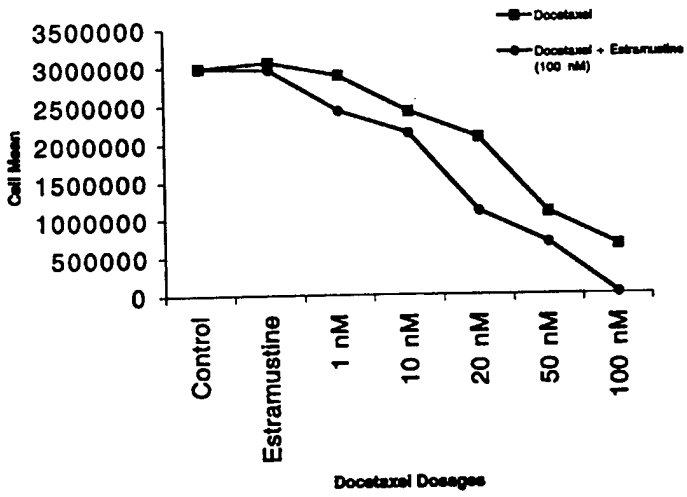

Fig. 2. MLL cells treated with Taxotere in vitro had an IC_{50} value of 40 nM. The addition of Emcyt (100 nM) lowered this value to approximately 15 nM.

RESULTS

In Vivo Cytotoxicity Experiments

The human prostate cancer cell line PC-3 and the Dunning rat R2237 metastatic anaplastic to lymph node, lung (MLL) subline were tested for sensitivity to docetaxel and estramustine in combination. The IC_{50} for docetaxel was approximately 2 nM for PC-3 cells (Fig. 1). The addition of 100 nM of estramustine did not significantly alter the IC_{50} for any docetaxel dose. In MLL cells, the IC_{50} was approximately 40 nM (Fig. 2). The addition of 100 nM of estramustine lowered the IC_{50} of docetaxel to approximately 15 nM.

In Vivo Animal Model

Copenhagen rats were injected with 1×10^6 MLL cells into each flank on day 0. Rats were treated intraperitoneally on days 4 and 11, and then sacrificed on day 14. There was a significant decrease (>50%) in tumor weight in mice treated with high dose docetaxel (Fig. 3). This was even more prominent in both low-dose docetaxel and high-dose docetaxel in combination with estramustine (100 nM). Clinically, decreased clearance of docetaxel was associated with estramustine combinations. However, clearance data were not obtained in this study.

DISCUSSION

The evaluation of new agents and combination therapies in metastatic prostate cancer has led to the realization of increased response rates due to synergism involving different mechanisms of action of various chemotherapy drugs on a related pathway (e.g., DNA synthesis, antimetabolites, cell adhesion). Reduction in the required dosages of individual drugs and potentially decreased toxic side effects is another advantage of using these combination therapies.

Estramustine has been shown to bind to microtubule-associated proteins and disrupt microtubule organization in vitro [9,10]. Estramustine is currently indicated in the palliative treatment of patients with metastatic and/or progressive prostate cancer, with a response rate of 5–17% as a single agent [11]. Its toxicities include fluid overload and thrombophlebitis.

Docetaxel, a semisynthetic taxoid, interferes with the microtubular network essential for mitotic and interphase cellular functions [12,13]. It binds to free tubulin and promotes the assembly of stable microtubules that cannot disassemble [12]. As a single agent, it has been shown to be effective in chemoresistant breast cancer [14]. Bone marrow suppression is a major dose-limiting toxicity.

Combining agents that are active against a particular tumor but do not have overlapping toxicities has been a basic rule in clinical oncology. In patients with prostate cancer, this has not been the case. Preclinical studies demonstrated that a single-agent estramustine (noneffective as a single agent) and docetaxel (noneffective as a single agent) demonstrated synergistic activity, and the combination of the two agents was clinically much more effective than when either agent was given alone [15].

A phase I trial evaluated Emcyt and Taxotere [16]. The regimen consisted of Emcyt 280 mg PO TID days 1–5, Taxotere 40, 60, or 70 mg/m^2, and Decadron 20 mg 6 + 12 hr, and 15 min prior to Taxotere on day 2. The cycle was repeated every 21 days. The overall response rate was 62%, as defined by a PSA decrease of at least 50%. Of the responders, 69% failed steroids and 54% failed Emcyt. Forty-three percent of patients with measurable disease had a partial response. The

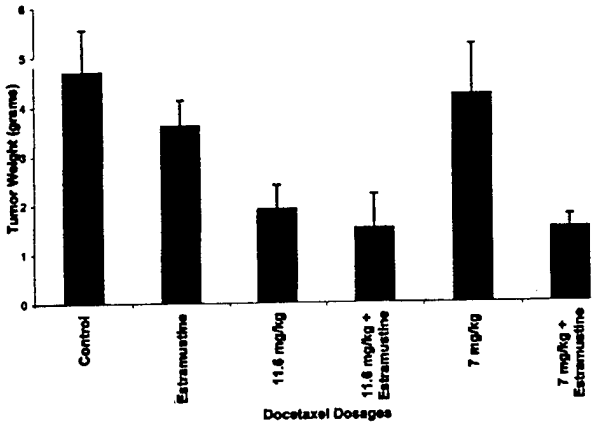

Fig. 3. In vivo study, demonstrating a decrease in tumor mass of mice treated with Taxotere (11.6 mg/kg) alone. The addition of Emcyt (100 nM) significantly lowered the tumor mass in mice treated with low-dose Taxotere (7 mg/kg), but not high-dose Taxotere (11.6 mg/kg), suggesting an additive effect in the latter.

toxicities included fluid retention, granulocytopenia, and hepatotoxicity.

Another phase I/II trial by Kreis in 1999 [15] again involved Emcyt and Taxotere. The regimen consisted of Emcyt 140 mg/10 kg body weight PO QD, Taxotere 40, 60, or 80 mg/m^2, and Decadron 8 mg BID × 5 doses. The cycle was repeated every 21 days. Nine patients in total were enrolled. Four patients had at least a 50% decrease in PSA. The toxicities included esophageal dyspepsia, nausea, and diarrhea.

Our results demonstrate that docetaxel and estramustine have a significant cytotoxic effect in the PC-3 and MAT-LyLu prostatic cell lines. We demonstrated enhanced cytotoxic effects when these drugs were used in combination, both in vitro and in vivo, with MLL cells. These results are similar to those reported by other authors using combinations of these agents.

CONCLUSIONS

In vitro, docetaxel has significant cytotoxic activity. This effect was also demonstrated in vivo in this study. The addition of estramustine did not enhance this effect in vitro in the PC-3 cell line, but was significant in MLL cells. In vivo studies demonstrated a significant decrease in tumor weight of mice treated with either low- or high-dose docetaxel in combination with estramustine.

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ELSEVIER

DIFFERENT DOCETAXEL-INDUCED APOPTOTIC PATHWAYS ARE PRESENT IN PROSTATE CANCER CELL LINES LNCaP AND PC-3

HEATHER J. MUENCHEN, PAUL J. PONCZA, AND KENNETH J. PIENTA

ABSTRACT

Objectives. To investigate the molecular machinery of docetaxel (Taxotere)-initiated death signaling on prostate cancer cell lines LNCaP and PC-3. Taxotere is a member of the taxane family of chemotherapeutic agents. It has been shown to disrupt microtubule dynamics causing mitotic arrest, which leads to cell death. Taxotere has demonstrated induction of cell death in LNCaP and PC-3 cells. However, the pathways by which apoptosis occurs differ in each cell line.

Methods. The prostate cancer cell lines, LNCaP and PC-3, were treated with 40 nM Taxotere for various lengths of time (0.5 to 24 hours). Western blot analysis was used for protein analysis.

Results. LNCaP cells demonstrated caspase-3 and caspase-7 cleavage, and PC-3 cells demonstrated only caspase-8 and BH3-interacting domain death agonist cleavage. Only LNCaP cells were observed to express clusterin expression; PC-3 cells expressed a novel apoptosis inhibitor, survivin.

Conclusions. In this study, we demonstrated two distinctly different Taxotere-induced apoptotic pathways in LNCaP and PC-3 cells that may be of clinical importance when treating prostate cancer. UROLOGY **57:** 366–370, 2001. © 2001, Elsevier Science Inc.

Docetaxel (Taxotere), a member of the taxane family, binds to beta-tubulin and promotes microtubule assembly and microtubule bundling.[1] This binding stabilizes the microtubule spindle and impairs mitosis, blocking progression through the cell cycle.[2] This taxoid has also been shown to phosphorylate the protein bcl-2, which inactivates bcl-2 to promote apoptosis.[3]

Phosphorylation of bcl-2 leads to the release of cytochrome c from the mitochondria and subsequent activation of an initiator caspase, caspase-9.[4] The initiation and/or execution of apoptosis after the lethal stimulus are typically signaled through the activation of caspases, proteins encoded by the *CASP* gene family.[5] Caspases are a unique family of cysteine proteases that differ in sequence, structure, and substrate specificity from any other protease family. This family, termed CED-3/caspase-1, was first identified in *Caenorhabditis elegans*.[6] The CED-3 death gene demonstrates high similarity to interleukin-1-beta-converting enzyme (ICE)[6] and is composed of caspase-1, caspase-2, caspase-3, caspase-4, caspase-6, caspase-7,[7] caspase-9,[8] and caspase 10.[9] These caspases are part of a cell's apoptotic machinery and act to destroy specific target proteins critical to cell survival. Caspase-3, caspase-7, and caspase-9 have been shown to cleave poly (adenosine diphosphate) polymerase from a 112-kDa nuclear protein into an 85-kDa apoptotic fragment.[8] Caspase-3 and caspase-7 have been shown to be critical mediators of apoptosis in LNCaP and TSU-Pr1 prostate cancer cells.[10] However, the caspase pathway of the androgen-independent PC-3 cell line has not yet been reported.

Clusterin, also known as testosterone-repressed message-2, was first isolated from ram rete testes fluid.[11] Clusterin functions include the transport of lipoproteins, inhibition of complement-mediated cell lysis, and modulation of cell-cell interactions.[12] Clusterin is also a putative marker for cells

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proceeding through apoptosis.[13] Other studies have demonstrated the presence of clusterin expression in both LNCaP and PC-3 cells.[14,15]

An anti-apoptosis gene, survivin, has also been implicated in prostate cancer. Survivin is a unique mammalian inhibitor apoptosis protein.[16] It is unlike that of other inhibitor apoptosis proteins, which are expressed throughout both adult and fetal tissues. Survivin is expressed only in the embryonic lung and other organs in developmental stages and is undetectable in normal adult tissues, other than the thymus and placenta.[16] Survivin is also expressed in transformed cell types and in most human cancers.[16]

We investigated the molecular basis of Taxotere-mediated apoptosis in LNCaP and PC-3 cells. Our data demonstrate two distinctly different Taxotere-induced apoptotic pathways for these prostate cancer cell lines.

MATERIAL AND METHODS

Cell Cultures

LNCaP (androgen-responsive) and PC-3 (androgen-independent) cell lines (American Type Culture Collection, Rockville, Md) were maintained at 37°C in an atmosphere of 5% carbon dioxide in RPMI 1640 medium (Life Technologies, Grand Island, NY) containing 1% antibiotic-antimycotic (penicillin G, 10,000 U/mL; streptomycin sulfate, 10,000 μg/mL; and amphotericin B, 25 μg/mL, Life Technologies) and supplemented with 10% fetal bovine serum (Life Technologies).

Treatments

Taxotere (Rhône-Poulenc Rorer, Collegeville, Pa) was prepared in normal media at a 40 nM concentration. Media from LNCaP and PC-3 cells, at 80% confluence, were removed, and fresh drug-containing media was added to the cultures. Protein from the cultures was isolated at 0, 0.5, 1, 2, 4, 8, 16, and 24 hours after treatment.

Cytotoxicity Assay

Taxotere cytotoxicity was evaluated using cell attachment as an indicator of cell viability. For the cell attachment assay, logarithmic-growing LNCaP and PC-3 cells were incubated with Taxotere (0 to 1 μM) in triplicate. Twenty-four hours later, the attached viable cells were counted by the Coulter counter model Z1, as previously reported.[17] Cell survival (percentage) was calculated as (experiment ÷ control) × 100%. The results were presented as the mean with the percentage of the standard error.

Cytosolic Extracts

Cell pellets were washed in phosphate-buffered saline, pelleted again, resuspended in buffer A (10 mM Hepes [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 5 mM dithiothreitol, 5 mM phenyl methyl sulfonyl fluoride, and protease inhibitors [50 μg/mL antipain, 2 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin]) and placed on ice for 10 minutes. The cells were then aggitated and centrifuged for 10 seconds. The supernatant was placed in a separate tube and 10 mM of ethylenediaminetetraacetic acid, 120 mM of KCl, and 20% glycerol was added. This cytosolic extract mixture was stored at −80°C.

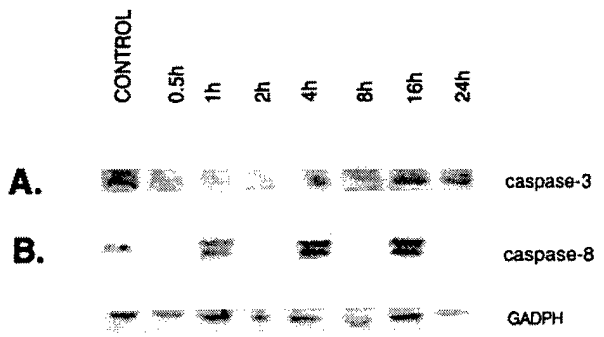

FIGURE 1. *(A) Caspase-3 is involved in Taxotere-induced cell death in LNCaP cells. LNCaP cells were treated with 40 nM of Taxotere for the indicated time points. Total cellular proteins (100 μg) were subject to SDS-PAGE followed by immunoblotting for caspase-3. (B) Caspase-8 is involved in Taxotere-induced cell death in PC-3 cells. PC-3 cells were treated with 40 nM of Taxotere for the indicated time points. Total cellular proteins (100 μg) were subject to SDS-PAGE followed by immunoblotting for caspase-8. GADPH was used as a control for both parts A and B.*

Western Blot Analysis

Equal amounts of cytosolic extract (100 μg) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Western blotting using polyclonal goat caspase-3, caspase-7, and caspase-8 antibodies (Santa Cruz Biotechnology, Santa Cruz, Calif), polyclonal goat BH3-interacting domain death agonist (BID), clusterin, and c-Fos antibodies (Santa Cruz Biotechnology), and polyclonal rabbit survivin antibody (R&D Systems, Minneapolis, Minn).

RESULTS

Cytotoxicity Assay of Taxotere on LNCaP and PC-3 Cells

When LNCaP and PC-3 cells were exposed to different dosages of Taxotere (0 to 1 μM), the LD_{50} (defined as the concentration of the agent needed to inhibit the growth of cells by 50%) was approximately 40 nM (data not shown). On the basis of these cytotoxicity results, we used a concentration of 40 nM to study the various apoptotic stimuli induced by Taxotere.

LNCaP and PC-3 Cells Use Different Caspases to Achieve Apoptosis

To demonstrate which caspases are involved in the death of Taxotere-treated LNCaP and PC-3 cells, we analyzed the various caspases involved in apoptosis. These included caspase-3, 7, and 8. LNCaP cells demonstrated caspase-3 cleavage (Fig. 1A) and faint bands of caspase-7 were visualized (data not shown); however, no caspase-8 was detectable by Western blot analysis. In PC-3 cells, caspase-8 cleavage was observed (Fig. 1B), and caspase-3 and caspase-7 were not visualized. These results suggest that the LNCaP and PC-3 cell lines

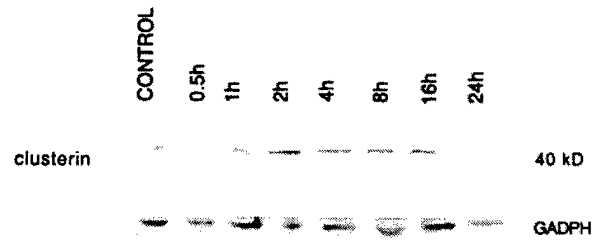

FIGURE 2. *Clusterin expression in LNCaP cells before and after treatment with 40 nM of Taxotere. Total protein was extracted from LNCaP cells at various time points and 100 μg was subject to SDS-PAGE followed by immunoblotting for clusterin.*

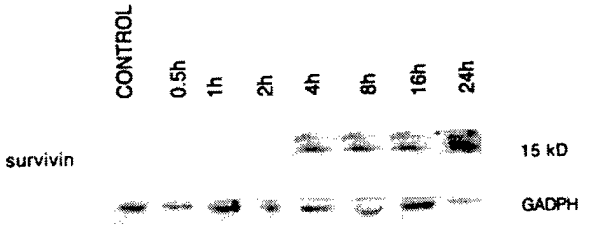

FIGURE 3. *Survivin is involved in Taxotere-induced cell death in PC-3 cells. PC-3 cells were treated with 40 nM of Taxotere for the indicated time points. Total cellular proteins (100 μg) were subject to SDS-PAGE followed by immunoblotting for survivin.*

use different caspases to proceed through Taxotere-induced apoptosis.

BID Cleavage in Taxotere-Treated PC-3 Cells

To examine the cleavage of BID, Western blot analysis was performed using total cellular protein. BID cleavage in PC-3 cells was seen after only 30 minutes of Taxotere treatment (data not shown). This cleavage was observed at all time points tested. LNCaP cells did not demonstrate BID cleavage. These data support our hypothesis that caspase-8 cleaves BID in PC-3 cells going through Taxotere-induced apoptosis.

Clusterin Expression in LNCaP Cells Is Absent in PC-3 Cells

Western blot analysis of clusterin expression in Taxotere-treated LNCaP cells demonstrated activity at all time points, except at 0.5 and 24 hours (Fig. 2). PC-3 cells did not show clusterin expression at any time points tested (data not shown). To support this finding, we also investigated c-Fos. Activated c-Fos has been shown to repress clusterin gene expression.[18] Phosphorylation, or activation, of c-Fos was seen in PC-3 cells at all time points, with the exception of 4 and 8 hours (data not shown). A faint band at 1 hour and the absence of a band at both the 4 and 8-hour time points demonstrated the half-life of c-Fos, which is approximately 2 hours. Taxotere treatment may have had an effect on the half-life of c-Fos; however, it did not affect its constitutive activation. LNCaP cells, however, demonstrated no activation of c-Fos at any point. These results suggest that expression of activated c-Fos in PC-3 cells represses clusterin gene expression and treatment of these cells with 40 nM of Taxotere does not induce clusterin expression.

Expression of a Novel Apoptosis Inhibitor, Survivin, in PC-3 Cells

Treatment of PC-3 cells with 40 nM of Taxotere resulted in increased expression of survivin at 4, 8, 16, and 24 hours after treatment (Fig. 3). The absence of bands at 0.5, 1, and 2 hours was due to the half-life of survivin, which, similar to c-Fos, is approximately 2 hours. LNCaP cells, however, did not demonstrate survivin expression at any point. These results suggest a novel pathway of apoptosis for the androgen-independent cell line, PC-3, different from that of the androgen-responsive LNCaP cell line.

COMMENT

The results of this present study suggest that the androgen-responsive LNCaP cells use a different apoptotic pathway than the androgen-independent PC-3 cells when treated with Taxotere. In this study, we demonstrated that LNCaP cells cleave caspase-3 after 30 minutes of Taxotere treatment (40 nM). Faint bands demonstrating caspase-7 were also visualized. No caspase-8 was detected before or after Taxotere treatment. However, the opposite was true in PC-3 cells. After treatment with 40 nM Taxotere, cleavage of caspase-8 was detected after only 1 hour. Neither caspase-3 nor caspase-7 could be visualized in this cell line before or after Taxotere treatment.

Caspase-8, also known as Mch5, MACHα1, or FLICE, exhibits a high sequence homology with caspase-3 and caspase-7.[19] However, caspase-8 is associated with the Fas-associated death domain,[20] which has been shown to serve as a docking protein for caspase-8, tethering the enzyme to activated Fas. The cross-linking of Fas to its cognate ligand results in the activation of signaling cascades, including the cleavage of caspase-8, which leads to cell death.[21] Caspase-8 has been associated with BID, a BH3 domain-containing pro-apoptotic bcl-2 family member.[22] Although BID is located within the cytosol, a truncated form of BID, cleaved by caspase-8, translocates to the mitochondria and transduces apoptotic signals from the cytoplasmic membrane to the mitochondria (Fig. 4).[22] Phosphorylation of bcl-2, release of cyto-

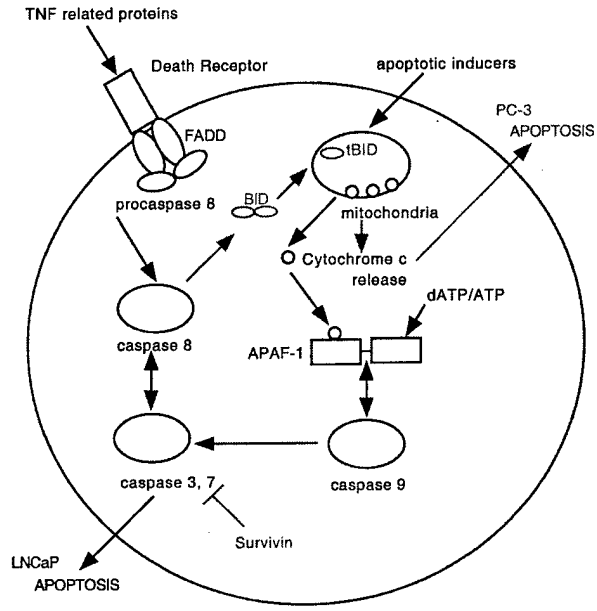

FIGURE 4. *The two different Taxotere-induced apoptotic pathways in LNCaP and PC-3 cells are shown. Although BID is located within the cytosol, a truncated form of BID (tBID), cleaved by caspase-8, translocates to the mitochondria and transduces apoptotic signals from the cytoplasmic membrane to the mitochondria. TNF = tumor necrosis factor; FADD = Fas-associated death domain; dATP/ATP = deoxyadenosine triphosphate/adenosine triphosphate; APAF-1 = apoptosis protease activating factor-1.*

chrome c, loss of mitochondrial membrane potential, cell shrinkage, and nuclear condensation are all induced by caspase-8 without the initiation of the caspase cascade.[22] This phenomenon explains the phosphorylation of bcl-2 without subsequent activation of caspase-3 and caspase-7 in PC-3 cells.

Survivin, a novel apoptosis inhibitor, is expressed constitutively in PC-3 cells. It is degraded 0.5, 1, and 2 hours after treatment with 40 nM Taxotere but appears again at the 4-hour point and is sustained, increasing in quantity through 8, 16, and 24 hours. Survivin has been shown to inhibit apoptosis by binding to the terminal effectors, caspase-3 and caspase-7, but not to their proforms or to the active initiator caspase-8.[16] It has also been implicated in protecting cells from anticancer drugs such as paclitaxel.[23] Our data suggest that the expression of survivin blocks the activation of caspase-3 and caspase-7 in PC-3 cells.

We also looked at an apoptosis maker previously found in prostate cancer cells.[14] Clusterin was expressed in LNCaP cells and increased in quantity after 2 hours of Taxotere treatment. However, clusterin was absent in PC-3 cells before and after treatment. To support this finding, we looked at c-Fos, which has been shown to repress clusterin expression.[18] Our data demonstrate phosphorylation of c-Fos in PC-3 cells, both before and after treatment with Taxotere. No activation of c-Fos was present in LNCaP cells and therefore clusterin is expressed. These data suggest that the activation of c-Fos in PC-3 cells represses clusterin expression.

CONCLUSIONS

Although both LNCaP and PC-3 cells represent special cases in prostate cancer biology, on the basis of the data presented here, we believe that the androgen-responsive cell line, LNCaP, proceeds through Taxotere-initiated apoptosis by way of phosphorylation of bcl-2, release of cytochrome c, and subsequent activation of the caspase cascade, which includes the initiator caspase-9, activating the effector caspases, caspase-3 and 7. Clusterin expression appears to be an appropriate indicator of apoptosis for these cells, as c-Fos is not activated and therefore not repressing its expression. On the contrary, PC-3 cells appear to use an entirely different pathway. The androgen-independent PC-3 cells do not express caspase-3 or caspase-7; however, they do demonstrate activation of caspase-8. These cells appear to proceed through apoptosis using caspase-8, which cleaves BID and phosphorylates bcl-2, causing release of cytochrome c and apoptosis of the cell, bypassing the caspase cascade. The survivin expression found in PC-3 cells supports this pathway, because of its blockage of caspase-3 and caspase-7 activation. These two distinctly different pathways deserve further study and will be included in our future investigations. That androgen-insensitive PC-3 cells undergo apoptosis through a different pathway opens up new directions for designing novel molecular targets for hormone-refractory prostate cancer.

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The regulation of prostate cancer cell adhesion to human bone marrow endothelial cell monolayers by androgen dihydrotestosterone and cytokines

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Abstract

A previous study from our laboratory suggested that prostate cancer metastasis to bone may be mediated, in part, by preferential adhesion to human bone marrow endothelial (HBME) cells. Tumor cell adhesion to endothelial cells may be modulated by the effect of cytokines on cell adhesion molecules (CAMs). Tumor necrosis factor-alpha (TNF-α) regulates VCAM expression on the endothelium and this effect is enhanced by dihydrotestosterone (DHT). Transforming growth factor-beta (TGF-β) stimulates the expression of $\alpha_2\beta_1$ integrin on PC-3 cells. The current study investigated the effects of the above cytokines and DHT (singularly and in various combinations) upon HBME and prostate cancer cell expression of VCAM, α_2 integrin subunit, and β_1 integrin subunit by flow cytometry. We also monitored the effects of the above treatments on PC-3 cell adhesion to HBME monolayers. The data demonstrate that none of the treatments significantly altered the expression of selected CAMs on HBME cell and neoplastic prostate cell lines. The treatment of HBME monolayers with various combinations of cytokines and DHT prior to performing adhesion assays with PC-3 demonstrates that treatments containing TGF-β reduced PC-3 cell adhesion to HBME monolayers by 32% or greater ($P < 0.05$). The reduction in PC-3 cell adhesion to TGF-β-treated HBME monolayers was dose dependent. Interestingly, LNCaP cells but not PC-3 cells treated with TGF-β had a reduced ability to adhere to untreated HBME monolayers. These results suggest that TGF-β may reduce tumor cell adhesion to bone marrow microvascular endothelium, *in vivo*. The biological significance of this observation is discussed.

Introduction

During cancer cell metastasis, tumor cells detach from the primary mass, enter blood circulation, adhere to vascular endothelial cells at a specific site, exit blood circulation at that site, induce angiogenesis, and establish a secondary mass [1]. Prostate cancer preferentially metastasizes to bone and causes significant bone pain [2]. A previous study suggested that prostate cancer metastasis to bone may be, in part, a result of preferential adhesion to human bone marrow endothelial (HBME) cells [3]. The cell adhesion molecules (CAMs) responsible for PC-3 cells' (a prostate cancer cell line derived from a bone metastasis) interaction with HBME cells, however, were not extensively examined in that study.

CAMs mediate, in part, the adhesion of tumor cells to the endothelium. The CAMs on endothelial cells are typically influenced by inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) [4, 5]. Endothelial cells stimulated with TNF-α express increased amounts of vascular cell adhesion molecule-1 (VCAM-1) and E-selectins. Both CAMs have been shown to mediate tumor cell adhesion to vascular endothelial cells [5–7]. Interestingly, the TNF-α-induced expression of VCAM-1 on human umbilical vein endothelial cells (HUVECs) is enhanced by treatment of HUVECs with dihydrotestosterone (DHT) [8].

The expression of CAMs on prostate cancer cells is also regulated by cytokines [9]. The integrin $\alpha_2\beta_1$ mediates PC-3 cell adhesion to collagen I, which is a major component of the bone matrix. Furthermore, the expression of this integrin on PC-3 cells is upregulated by transforming growth factor-beta (TGF-β), which is a prevalent cytokine in the bone marrow. TGF-β first binds TGF-β receptor type II, which presents the TGF-β ligand to TGF-β receptor type I. The TGF-β receptor type I is activated by phosphorylation and interacts with an adaptor protein SARA (Smad anchor for receptor activation). SARA then propagates the TGF-β signal to intracellular signaling mediators known as Smad2 and Smad3. Smad 2 and 3 interact with Smad 4 and the Smad complexes are translocated to the nucleus, where they activate specific genes. Interestingly, Smad 3 is an important

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coregulator for androgen-signaling in prostate cancer cells [10].

Nakashima et al. [11] reported that serum levels of TNF-α are elevated in prostate cancer patients. TNF-α facilitates metastasis by enhancing endothelial cell expression of CAMs that would in turn mediate cancer cell adhesion to the endothelium [7]. In addition, the normal physiological levels of DHT (4–40 nmol/l) can enhance surface expression of VCAM on TNF-α stimulated endothelial cells [8]. TGF-β is a prevalent cytokine in the bone matrix and has been demonstrated to enhance the expression of the collagen I receptor, $\alpha 2\beta 1$ [12]. It is therefore conceivable that TGF-β regulates CAM expression on both prostate cancer cells and HBME cells that can affect their interaction.

Based upon these findings, we examined the interplay between CAMs, cytokines and androgens such as DHT. Specifically, the present study investigated the effects that TGF-β, TNF-α, and DHT (singularly and in various combinations) have upon selected prostate cancer cell adhesion to HBME cell monolayers. We also determined the effect these soluble factors have on HBME cell expression of three CAMs: $\alpha 2$ integrin subunit, $\beta 1$ integrin subunit, and VCAM. Lastly, we investigated the effects of these soluble factors on the surface expression of $\alpha 2$ integrin and $\beta 1$ integrin subunits on normal and neoplastic prostate cell lines. TNF-α was excluded from the treatment protocol for normal and neoplastic prostate epithelial cell lines because it has a cytotoxic effect on some cancer cell lines in this study [13].

Materials and methods

Cell lines

Human bone marrow endothelial (HBME) cells were developed and described in a previous publication [3]. Normal prostate epithelial cells, 267B1, were a generous gift from Dr Jill Mascoska, University of Michigan Comprehensive Cancer Center (Ann Arbor, Michigan). WiDr (colon cancer cell line), LNCaP (prostate cancer cell line derived from a lymph node metastasis that expresses androgren receptor) and PC-3 (prostate cancer cell line derived from a bone metastasis that does not express androgren receptor) were obtained from the American Type Culture Collection (ATCC). HBME and WiDr were maintained in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin; LNCaP and PC-3 were maintained in RPMI Medium with 10% FBS and 1% penicillin/streptomycin. The immortalized normal prostate epithelial cell line, 267B1, was maintained in Defined Keratinocyte-SFM supplemented with growth factors (Gibco), 10% FBS and 1% penicillin/streptomycin.

Flow cytometry procedure and analysis

The cell lines were seeded in either T-75 or T-150 flasks and grown to confluence. Once confluent, the HBME cell monolayers, normal and neoplastic epithelial cells were treated as described in respective figure legends. The concentrations of DHT and TNF-α were used because they were demonstrated to increase the expression of VCAM on endothelial cells [8]. TGF-β concentration was selected because it increased PC-3 adhesion to bone matrix [12]. Before flow cytometric procedures, one million cells were incubated with 100 μl of antibodies to α_2 integrin subunit, β_1 integrin subunit or VCAM (for HBME cells only) at a concentration of 10 μg/ml for 45 min at room temperature. The cells were washed with 10 volumes of PBS, centrifuged and the pellets were incubated with 10 μg/ml of anti-mouse FITC conjugate IgG (Sigma Chemical Co., St. Louis, Missouri) for 45 min at room temperature. Cells incubated with secondary antibodies only served as negative controls. After incubation with secondary antibodies, cells were washed again with 10 volumes of PBS, fixed with 3% paraformaldehyde, pH 7.4, and analyzed for fluorescence with an Epics Elite Flow Cytometer (Coulter Corp., Miami, Florida). The percentage of positive cells was determined by subtracting the negative control value of the histogram from tested sample values. The average of two replications is reported.

Adhesion assays

Adhesion assays were performed as reported earlier [3]. Briefly, human bone marrow endothelial (HBME) cells were seeded onto plastic and grown to confluence in 'snap-apart' 96-well tissue culture plates (Fisher Scientific, Pittsburgh, Pennsylvania). Prostate cancer cells (PC-3 or LNCaP) or HBME cells were treated with various combinations of cytokines and DHT for approximately 24 h prior to the adhesion assay. The media containing the treatments were removed shortly before starting the adhesion assay and replaced with adhesion media (MEM, 1% bovine serum albumin). Prostate cancer cells were labeled with ^{51}Cr sodium salt and layered over monolayers of HBME cells for 30 min at 37 °C. The plates were gently washed three times with PBS and assayed for gamma particle emission using a gamma counter. Cell adhesion was reported as counts per minute (CPM) relative to the adhesion of controls, which were set to 100. Experiments were done in quadruplicates and repeated a minimum of two times.

Membrane preparation and Western blotting

Cells were dounced in a 20 mM HEPES buffer (pH 7.4) supplemented with 5 mM Na_3VO_4 and 1 mM phenylmethylsulfonylflouride (PMSF). The resulting slurry was centrifuged at 100 000 $\times$ g for 35 min at 4 °C to pellet membrane fractions. The membrane fractions were solubilized in lysis buffer (20 mM sodium phosphate (pH 7.5), 100 mM NaCL, 1% triton X-100, 0.5% Na^+ deoxycholate and 0.1% SDS) and the protein concentrations were determined. Fifty micrograms of protein were resolved on a 7.5% polyacrylamide ready gel and transferred to a nitrocellulose membrane in accordance with manufacturer's instructions (Bio-Rad Laboratories, California). The membrane was blocked overnight at 4 °C with blocking buffer (Tris-buffered saline (TBS) pH 7.7 {0.05M Tris base, 0.15M NaCL} supplemented with 0.05% tween 20 and 10% nonfat powdered milk), probed for

1 h at room temperature (RT) with rabbit polyclonal anti-TGF-β type II receptor (Santa Cruz Biotechnology) diluted 1/5000 in probing solution (TBS pH 7.7 supplemented with 0.1% tween 20 and 2.5% nonfat powdered milk) and washed four times with probing solution. Next, the membrane was subjected to goat anti-rabbit IgG secondary antibody diluted 1/1000 in probing solution for 1 h at RT, washed four times and band visualized by chemiluminesence according to manufacturer's instructions.

Statistical analysis

For flow cytometry data, equality among treatment effects were assessed by Kruskal–Wallis tests due to the small number of replications for each treatment. For adhesion assay data, natural-log transformed measurements were analyzed by analysis of variance (ANOVA) to assess treatment effects. Adjustments for experimental groups were made where applicable. The estimates represent the expected values of the experimental group relative to the control group. In the graphs, the control group is plotted at 100. The estimated value and its 95% confidence interval were obtained by taking the anti-logarithm of the results from the analysis of variance. Statistical analyses were carried out with the SAS 8 software.

Results

The effects of cytokines and DHT on prostate cancer cell adhesion to HBME monolayers

Adhesion assays were done to determine the effect of soluble factors on HBME cell monolayers' ability to bind PC-3 cells. The data demonstrates a 39% reduction (95% confidence interval (22%, 52%) in PC-3 cell adhesion to HBME cells only when the treatment included TGF-β1 (Figure 1, $P = 0.001$). Neither TNF-α nor DHT, alone or in combination, affected PC-3 cell adhesion to HBME cell monolayers. To determine if the observed decrease in PC-3 cell adhesion to TGF-β-treated HBME cells is dose dependent, HBME cells were treated with various doses of TGF-β and then used in adhesion assays. The data demonstrate that the adhesion of PC-3 cells to TGF-β-treated-HBME cell monolayers is dose-dependent beyond a concentration of 0.1 ng/ml (Figure 2, $P = 0.001$).

To determine the effect TGF-β had on PC-3 cells' and LNCaP cells' ability to adhere to HBME monolayers, PC-3 and LNCaP cells were treated with 10 ng/ml of TGF-β. This concentration was demonstrated to increase PC-3 cell adhesion to collagen type I enriched matrix [9]. Our result demonstrates that treatment of LNCaP cells ($P = 0.02$), but not PC-3 cells ($P = 0.14$), with TGF-β significantly reduces their ability to adhere to HBME cell monolayers (Table 1). Western analysis was done on membrane preparations from cell lines used in this study to evaluate the expression of TGF-β receptor type II, which binds TGF-β ligand from the culture fluid [14]. Beta-1 integrin subunit was probed as control for membrane preparations. The result demonstrates that TGF-β receptor type II is only expressed in the WiDr and LNCaP cell lines (Figure 3). As expected, all cell lines express beta-1 integrin subunit, thus demonstrating the success of our procedure for isolating membrane-associated proteins.

Table 1. The effect of TGF-β treatment on PC-3 and LNCaP adhesion to HBME cell monolayers.

Cell lines/treatment	Percentage relative to control, 95% confidence interval
LNCaP /Control	100
LNCaP/Treated	56 (36, 87)
PC-3/Control	100
PC-3/Treated	88 (74, 104)

Percent relative to controls of LNCaP and PC-3 cell adhesion to HBME monolayers.
Both LNCaP and PC-3 cells were treated for 24 h with 10 ng/ml of TGF-β prior to being used in adhesion assays.

Table 2. The effect of TNFα on endothelial CAM expression.

Antibodies	Non-stimulated	TNFα-stimulated
E-selectin	2	45
VCAM	3	65
PeCAM	99	–

HUVEC were probed for the above cell adhesion molecules under non-stimulated and cytokine stimulated conditions. Maximal expression of E-selectin was detected at 4 h of cytokine stimulation and VCAM maximal expression was detected at 16–24 h of cytokine stimulation. PeCAM does not require cytokine activation and normally found in high amount on endothelial cells. The numbers are percentage above background. Background was determined by the amount of fluorescence associated with cells untreated with antibodies.

The role of DHT in prostate cancer cell adhesion to HBME was next determined. Unlike PC-3 cells, LNCaP cells are androgen responsive. The effect of DHT on the proliferation of LNCaP cell is known [15]. To the authors' knowledge, however, no information exists regarding the effect DHT has on LNCaP cells' ability to adhere to an endothelial monolayer. Using a broad range of DHT concentrations in standard medium supplemented with charcoal-stripped fetal bovine serum, we demonstrate that DHT does not reduce LNCaP cell adhesion to HBME monolayers (Figure 4a). DHT only marginally decreases PC-3 cell adhesion at the highest dose (200 nmolar), which is above the normal physiological range [9] (Figure 4b). Because PC-3 cells do not express androgen receptors, this outcome was not expected and cannot be explained.

The effects of cytokines and DHT on CAM expression

HBME cells were treated with cytokines and DHT as described in Figure 1 for the expression of VCAM-1, α_2 and β_1. The concentration of DHT used in this study is within the normal range of testosterone in human male serum. The data

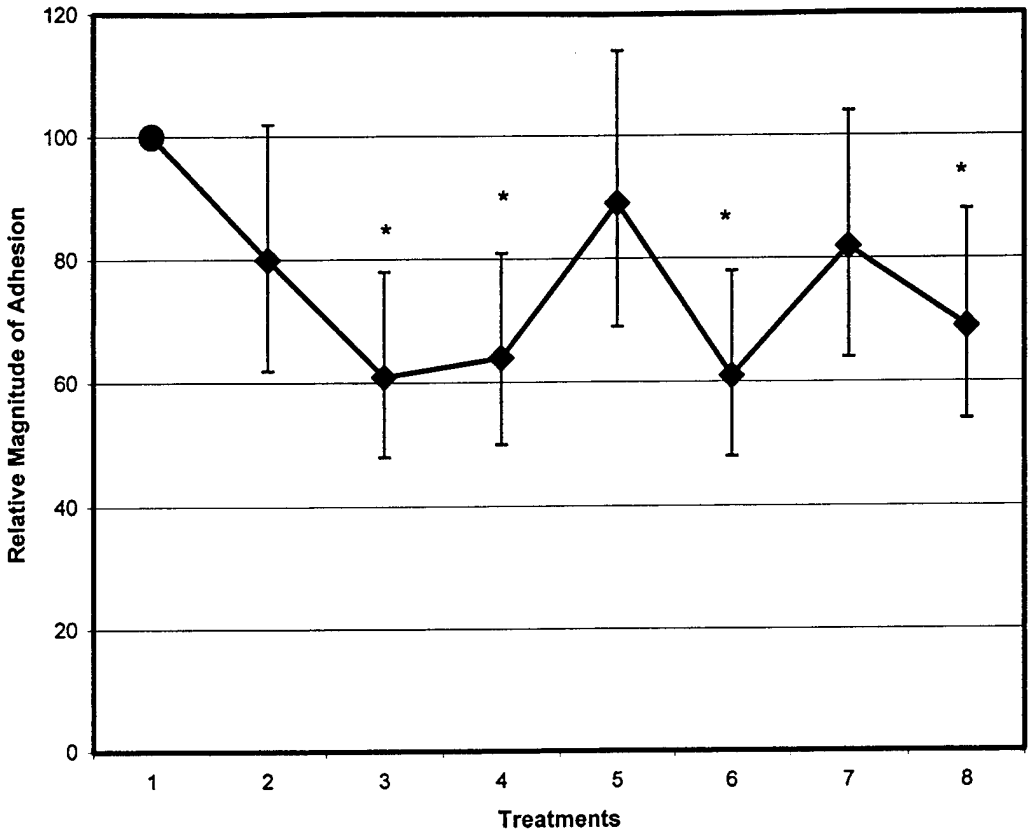

Figure 1. PC-3 cell adhesion to HBME cell monolayers treated with cytokines and DHT. The control (solid circle) is the magnitude of PC-3 cell adhesion to untreated HBME cell monolayer, which is defined as treatment 1. Treatments are as follows: (1) HBME culture media (Control), (2) 500 U/ml of TNF-α, (3) 10 ng/ml of TGF-β, (4) 500 U/ml of TNF-α and 10 ng/ml of TGF-β, (5) 40 nmol/l of DHT and 500 U/ml of TNF-α, (6) 40 nmol/l of DHT and 10 ng/ml, (7) 40 nmol/l of DHT, and (8) 40 nmol/L of DHT, 10 ng/ml of TGF-β, and 500 U/ml of TNF-α. The original culture medium was used as a solvent for each treatment. HBME cells were treated for approximately 24 h. Statistically significant values relative to the control are indicated by *($P < 0.05$). Bars represent 95% confidence interval based on the ANOVA.

demonstrate no statistically significant difference between the 8 experimental conditions for surface expression of VCAM, alpha-2 integrin subunit, and beta-1 intregin subunit (all P-values are > 0.17) (Figure 5). HUVEC monolayers were treated as a control for TNF-α effect on endothelial cells (Table 2). HUVEC are sensitive to TNF-α stimulation and respond by expressing VCAM and E-selectin. Our data demonstrate that VCAM and E-selectin expression on HUVECs are upregulated by TNF-α treatment, thus indicating that our TNF-α treatment is effective.

The effect of TGF-β and DHT on α_2 and β_1 integrin subunit expression in normal and neoplastic cell lines was investigated. TNF-α was excluded from treatment regimens because of its reported cytotoxity on the LNCaP cell line [13, 16]. The data demonstrate that none of the four treatments significantly affected the expression of α_2 and β_1 integrin subunits in any cell line evaluated (all P-values > 0.14) (Figures 6A and B).

Discussion

The role that important cytokines (i.e., TNF-α and TGF-β) and androgen may play in prostate cancer cell adhesion to HBME monolayers has not been studied well. Cytokines have been demonstrated to regulate the expression of CAMs on both endothelial cells and prostate cancer cells [12, 17]. The cytokine activation of some endothelial cell types is enhanced in the presence of DHT [8]. The current study reports the effect of DHT and selected cytokines on endothelial cells and prostate cancer cells, and gives a rationale for the cytokines that were evaluated.

Serum levels of TNF-α have been demonstrated to be elevated in patients with advanced prostate cancer [11]. TNF-α is a potent activator of CAM expression on endothelial cells [17]. CAMs typically expressed on endothelial cell surfaces in response to TNF-α stimulation are VCAM, E-selectin and intracellular cell adhesion molecule (ICAM) [17]. These CAMs are known to mediate adhesion of a variety of tumor cells to HUVEC [7, 18, 19]. TNF-α induced VCAM expression on HUVEC is enhanced in the presence of physiologic concentrations of DHT [8]. DHT alone does not induce VCAM expression. The current study demonstrates that our immortalized HBME cells do not express VCAM in response to TNF-α stimulation. This is not the case for HBME cells immortalized in other laboratories [17, 20]. Interestingly, prostate cancer cell lines used in this study do not express receptors for VCAM, ICAM and E-selectin [21, 22]. Galectin-3, which is carbohydrate-binding protein, is expressed in PC-3 and LNCaP cells and it has been demonstrated to mediate PC-3 cell adhesion to HBME monolayers [4, 23]. Although the receptor for

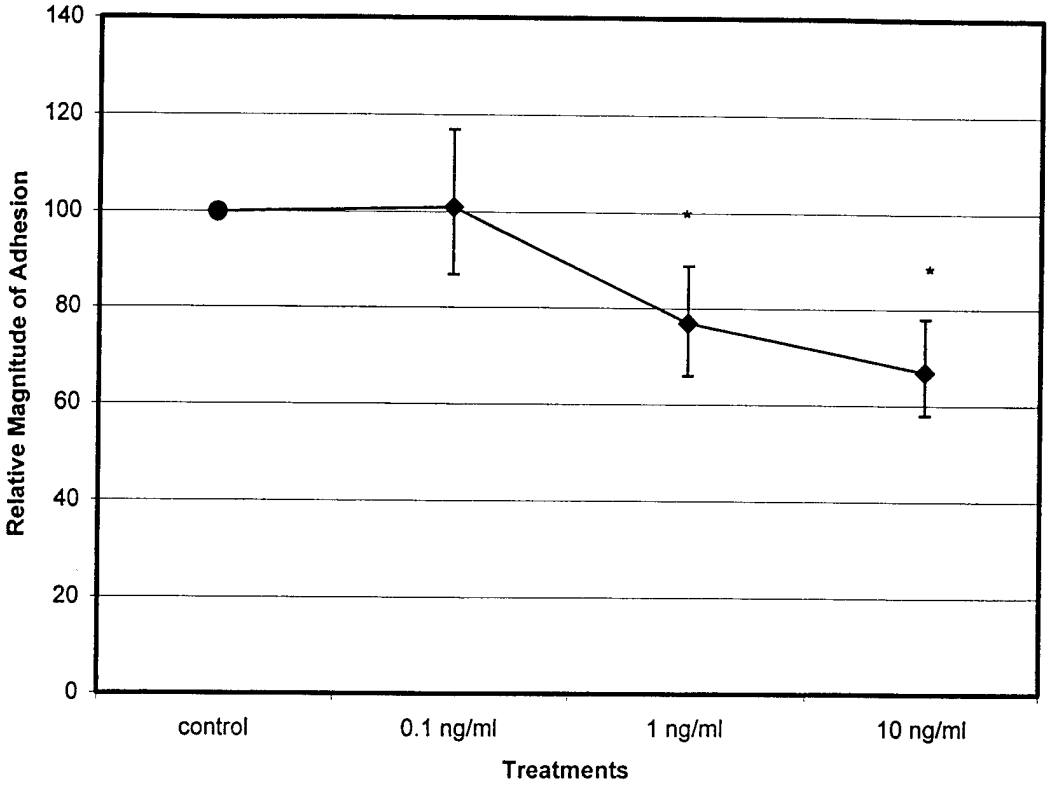

Figure 2. The effect of ascending concentrations of TGF-β on PC-3 cell adhesion to HBME cell monolayers. The control (solid circle) is the magnitude of PC-3 cell adhesion to untreated HBME cell monolayers. Statistically significant values relative to the control are indicated by *($P < 0.05$). Bars represent 95% confidence interval based on the ANOVA.

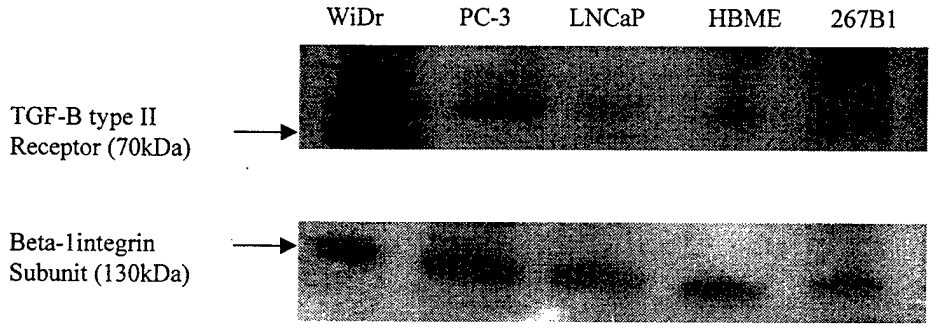

Figure 3. Western blot analysis of TGF-β receptor type II in membrane preparations of selected human cell lines. Beta-1 integrin subunit was evaluated as a control for the membrane associated proteins.

galectin-3 has not been identified, the data demonstrate that TNF-α stimulation does not alter its expression. The data generated during this investigation, therefore, suggest that TNF-α stimulation does not mediate prostate cancer cell adhesion to HBME cell monolayers via well-known inducible endothelial CAMs or unknown CAMs.

Although concentrations of plasma TGF-β do not correlate with progression of prostate cancer [23], this cytokine is prevalent in the bone, the preferred metastatic site for prostate cancer [24]. Kostenuik et al. [12] demonstrated that TGF-β upregulated PC-3 expression of $\alpha_2\beta_1$ and subsequently upregulated PC-3 cell adhesion to collagen type I. Data presented here does not support this phenomenon. Flow cytomeric analysis demonstrated that TGF-β did not alter $\alpha_2\beta_1$ expression on PC-3 cells and several cell lines including a colon cancer cell line (WiDr), a prostate cancer cell line derived from a lymph node metastasis (LNCaP), a normal prostate epithelial cell line immortalized with SV40 (NP,267B-1) [25] and our immortalized HBME cells. The difference between Kostenuik et al.'s study and the current study is that cell lysates were probed for $\alpha_2\beta_1$ in the former study. In the latter study, flow cytometry was used to evaluate $\alpha_2\beta_1$ expression at the cell surface. The inability of TGF-β to induce any response in selected cell lines was not due to the presence or absence of TGF-β receptor type II. We focused solely on receptor type II because it is responsible for binding TGF-β ligand from the medium and activating the signaling pathway [10]. TGF-β type II receptors were detected in WiDr and LNCaP cell membrane preparations by Western blotting. A previous study demonstrated that LNCaP and PC-3 cells expressed TGF-β receptor type II [15]. A more recent study [26] demonstrated that LNCaP cells do not express TGF-β receptor type II under conventional cell culture conditions. The objectives of these

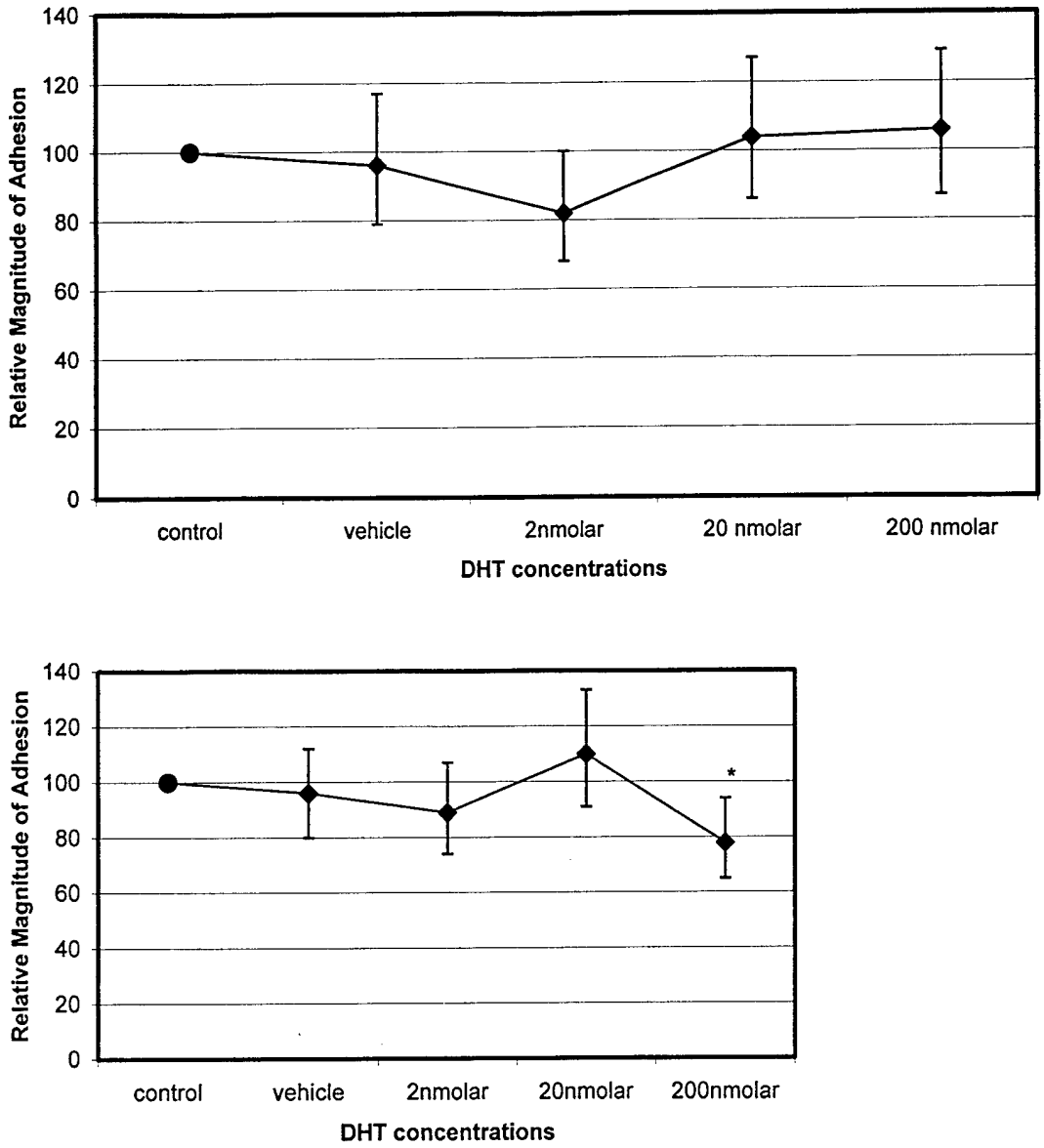

Figure 4. The effect of DHT on prostate cancer cell adhesion to HBME cell monolayers. A. LNCaP cells (A) and PC-3 cells (B) were treated for 24 h with increasing nanomolar concentrations of DHT dissolved in standard culture medium supplemented with charcoal-stripped fetal bovine serum. The control (solid circle) is the magnitude of untreated LNCaP cell adhesion to HBME cell monolayers. Statistically significant values relative to the control are indicated by $^*(P < 0.05)$.

particular experiments were (1) to determine if TGF-β up-regulated $\alpha_2\beta_1$ integrin expression in PC-3 cells only and (2) to determine if $\alpha_2\beta_1$ integrin plays a role in prostate cancer cell preferential adhesion to HBME *in vitro*. Alpha-2, beta-1 integrin mediates cell adhesion to extra-cellular matrix (ECM) components collagen type I, II, III and IV [22]. However, it is known that CAMs that mediate cell-to-ECM interactions can also mediate cell-to-cell interactions such as $\alpha_5\beta_1$ mediating the adhesion of human osteosarcoma cells, human melanoma cells and human kidney carcinoma cells to HUVEC [27]. The ligand for $\alpha_5\beta_1$ integrin is ECM component fibronectin [22]. Our data demonstrate that $\alpha_2\beta_1$ integrin is equally expressed on LNCaP, normal prostate epithelial cells and PC-3 cells, and slightly more expressed on the WiDr cells. WiDr cells adhere poorly to HBME cell monolayers and are used as a negative control in our adhesion assays [3]. Based on these observations, we conclude that $\alpha_2\beta_1$ integrin does not mediate preferential adhesion of prostate cancer cells to HBME cell monolayer.

The current study demonstrates that treatment of HBME monolayers with TGF-β significantly reduces their ability to bind PC-3 cells. The TGF-β treatment of LNCaP and not PC-3 cells also reduces their ability to bind HBME monolayers. This occurred despite our inability to detect TGF-β type II receptors on HBME cells. The TGF-β mediated inhibition was first demonstrated in a murine model consisting of P815 mastocytoma and microvascular endothelial cells [28]. The CAMs regulated by TGF-β were not identified in that study or the current study. The amount of TGF-β in the bone is greater than in any other organ [12]. Based on Bereta et al.' [28] and our observations, its presence in the bone should reduce the incidence of bone metastasis. This suggests that

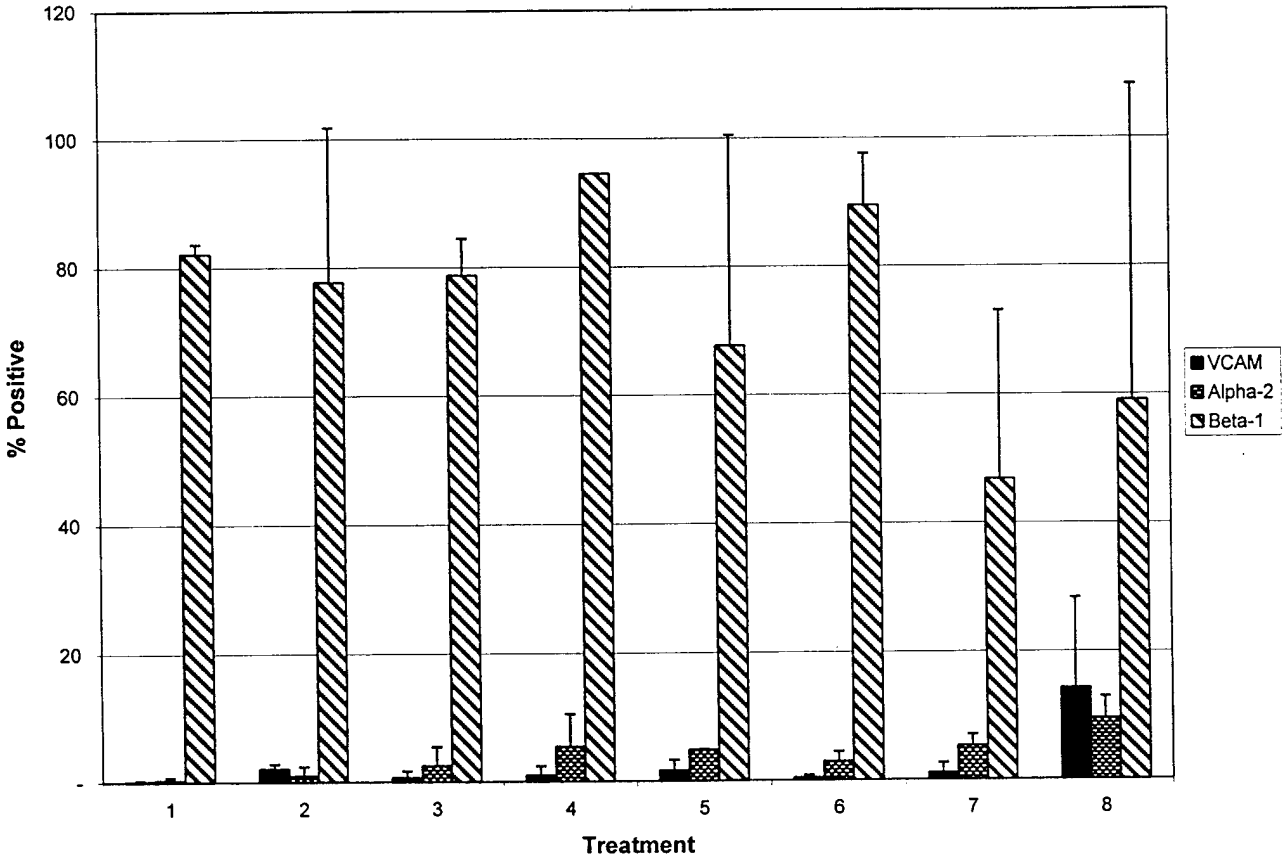

Figure 5. The effect of selected cytokine and DHT treatments on $\alpha_2\beta_1$ and VCAM expression on HBME cells as determined by flow cytometry. Treatments are as follows: (1) HBME culture media (Control), (2) 500 U/ml of TNF-α, (3) 10 ng/ml of TGF-β, (4) 500 U/ml of TNF-α and 10 ng/ml of TGF-β, (5) 40 nmol/l of DHT and 500 U/ml of TNF-α, (6) 40 nmol/l of DHT and 10 ng/ml, (7) 40 nmol/l of DHT, and (8) 40 nmol/l of DHT, 10 ng/ml of TGF-β, and 500 U/ml of TNF-α. The original culture medium was used as a solvent for each treatment. HBME cells were treated for approximately 24 h, the medium was removed and replaced with adhesion assay medium.

other factors in the bone may be negating the TGF-β effect on bone marrow endothelial cells and/or the concentration of TGF-β in the bone is not sufficient to alter CAM expression on the endothelium or metastasizing prostate cancer cells. Our data demonstrate a reduction in PC-3 cell adhesion to HBME cell monolayers at TGF-β concentrations exceeding 0.1 ng/ml. Using a murine model, Bereta and colleagues [28] demonstrated a reduction at TGF-β concentrations exceeding 0.5 ng/ml. Another explanation may be that TGF-β reduces bone metastasis in cancer cells that are TGF-β responsive, like LNCaP. The ability of TGF-β to reduce LNCaP but not PC-3 cell adhesion to HBME monolayers may explain the inability of LNCaP cells to metastasize to bone. Because TGF-β reduces HBME monolayers' ability to bind PC-3 but enhances PC-3's ability to bind collagen I, this cytokine may have mediated PC-3's ability to detach from the bone marrow endothelium and attach to the underlying bone matrix, which is composed mainly of collagen I [12].

In summary, this investigation demonstrates that DHT and TNF-α do not alter prostate cancer cell adhesion to bone marrow endothelial cells *in vitro*. Although TNF-α induces the expression of VCAM and E-selectin on HUVEC, it does not induce the expression of these CAMs on our established HBME cell line. HBME cell monolayers treated with TGF-β have a reduced affinity for prostate cancer cells *in vitro*. The TGF-β mediated effect is dose dependent. Because TGF-β is a common cytokine in the bone microenvironment, which is a common site for prostate cancer metastasis [12], it is unlikely that the effect of TGF-β on bone marrow endothelial cells prevents metastasis. We therefore speculate that TGF-β may play a role in releasing the attached cancer cells from the endothelium and upregulating their adhesion to the collagen I enriched bone matrix via enhanced $\alpha_2\beta_1$ expression [9, 12]. The prevalence of TGF-β in the bone and its ability to reduce endothelial cell binding to prostate cancer cells may be exploited to prevent skeletal complications associated with metastatic prostate cancer. Studies are currently underway to identify the CAMs on HBME cells that are regulated by TGF-β.

Acknowledgements

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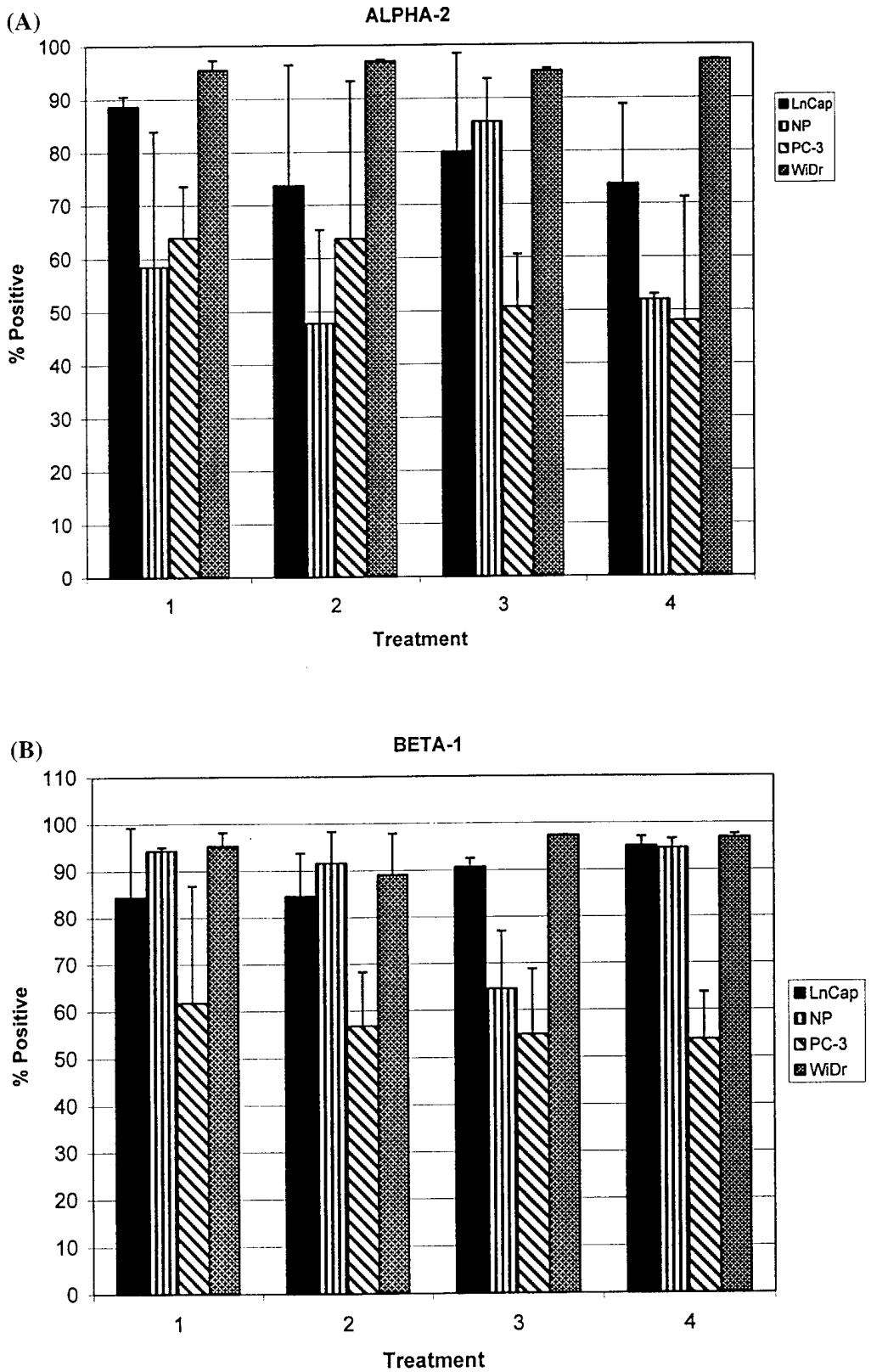

Figure 6. The effect of TGF-β and DHT treatments on $\alpha_2\beta_1$ expression in selected cell lines. A) Flow cytometric analysis of the $\alpha 2$ intregin subunit. B) Flow cytometric analysis of the $\beta 1$ intregin subunit. NP: normal prostate epithelial cell. 267B1. Treatments are as follows: (1) respective culture media (Control) for each cell lines, (2) 10 ng/ml of TGF-β, (3) 40 nmol/l of DHT, and (4) 40 nmol/l of DHT and 10 ng/ml. The original culture medium was used as a solvent for each treatment. HBME cells were treated for approximately 24 h, the medium was removed and replaced with adhesion assay medium.

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#4045 EXPRESSION OF CLUSTERIN IN ANDROGEN INDEPENDENT PROSTATE CANCER CELLS AFTER TRANSFECTION WITH AN IκBα "SUPER-REPRESSOR". Heather J Muenchen, D. L Linn, M. A Walsh, E. T Keller, and K. J Pienta, *Univ of Michigan, Ann Arbor, MI*

Sulfated glycoprotein-2 (SGP-2), also known as clusterin, has been associated with programmed cell death. In prostate cancer, SGP-2 expression is associated with castration-induced programmed cell death, and is also known as the testosterone repressed message (TRPM-2). These findings suggest that SGP-2 is a marker for cell death. Many androgen insensitive prostate cancer cells do not proceed through TNF-α- induced cell death due to the expression of antiapoptotic genes located in the nuclear factor-κB (NF-κB) family of transcription factors. NF-κB stimulates gene transcription when expressed in the nucleus, however, in resting cells, this nuclear import is prevented by association with the cytoplasmic inhibitor IκBα. During normal TNF-α cell activation, nuclear translocation of NF-κB is preceded by phosphorylation and degradation of IκBα. In these studies, we have transfected androgen dependent and independent prostate cancer cells with an IκBα "super-repressor", pCMV4-$I\kappa B_{S32A + S36A}$ which is a mutated nondegradable IκBα resistant to phosphorylation and degradation. This "super-repressor" mediated sensitization to TNF-α overcomes the TNF-α insensitivity of androgen independent cells and forces them through apoptosis. We have shown here that PC-3 cells transfected with pCMV4-$I\kappa B_{S32A + S36A}$ and stimulated with TNF-α (10ng) demonstrate production of SGP-2, where untransfected and control cells do not. Androgen dependent LNCaP cells express SGP-2 constitutively, but demonstrate breakdown of the protein into its subunits after pCMV4-$I\kappa B_{S32A - S36A}$ transfection and TNF-α stimulation.

436

TNF-α INSENSITIVE PROSTATE CANCER CELLS EXPERIENCE APOPTOSIS WHEN TRANSFECTED WITH AN IκBα "SUPER-REPRESSOR". *H. J. Muenchen, D. L. Lin, M. A. Walsh, E. T. Keller, K. J. Pienta.* Ann Arbor, MI. (Presented by H. J. Muenchen)

INTRODUCTION AND OBJECTIVES: Prostate cancer patients experiencing a relapse in disease often express high serum tumor necrosis factor-α (TNF-α) levels. In most cell systems, high levels of TNF-α would induce a cell to proceed through apoptosis. However, many androgen insensitive prostate cancer cells are TNF-α insensitive due to the expression of antiapoptotic genes located in the nuclear factor-κB (NF-κB) family of transcription factors. NF-κB stimulates gene transcription when expressed in the nucleus, however, in resting cells, this nuclear import is prevented by association with the cytoplasmic inhibitor IκBα. Low levels of TNF-α uncouples this cytoplasmic retention of NF-κB.During normal cell activation, nuclear translocation of NF-κB is preceded by phosphorylation and degradation of IκBα. When phosphorylation is blocked IκBα remains intact, thereby, blocking NF-κB translocation to the nucleus and subsequent activation of antiapoptotic genes that cause TNF-α insensitivity. We transfected a "super-repressor", which is a mutated nondegradable IκBα resistant to phosphorylation and degradation. into prostate cancer cells. This IκBα "super-repressor", pCMV4-IκB$_{S32A+S36A}$, blocked the nuclear translocation of NF-κB by TNF-α. Moreover, pCMV4-IκB$_{S32A+S36A}$ mediated sensitization to TNF-α overcame the TNF-α insensitivity of androgen independent cells and forced them through apoptosis.

METHODS: PC-3 and LNCaP cells were stimulated with TNF-α (10 ng/ml) for 24 hours in the presence or absence of (pCMV4-IκB$_{S32A+S36A}$) or the empty vector p6R as a control. NF-κB activity was measured by EMSA and the steady state levels of the cytoplasmic IκBα protein were measured by Western blot. Secretory IL-6 and IL-6 mRNA were measured by ELISA.

RESULTS: pCMV4-IκB$_{S32A+S36A}$ blocked the stimulation of NF-κB activity by TNF-α in prostate cancer cells. This "super-repressor" blocked the subsequent nuclear translocation of NF-κB and induction of IL-6 by TNF-α. Moreover, the pCMV4-IκB$_{S32A+S36A}$ mediated sensitization to TNF-α overcame the TNF-α insensitivity of androgen independent cells and forced them through apoptosis.

CONCLUSIONS: We conclude that this data demonstrates that inhibition of NF-κB selectively sensitizes previously insensitive prostate cancer cells to TNF-α.

Support: This work was supported by CaPCURE and SPORE grant P50CA69568

#774 The Role of Androgen and Cytokines in Prostate Cancer Cell Adhesion to Human Bone Marrow Endothelial Cell Monolayers. Carlton R. Cooper, Jasmine Bhatia, Heather J. Muenchen, Lisa Mclean, Paul Poncza, and Kenneth J. Pienta. *University of Michigan, Ann Arbor, MI.*

A previous study from our laboratory suggested that prostate cancer metastasis to bone may be mediated, in part, by preferential adhesion to human bone marrow endothelial (HBME) cells. The adhesion of tumor cells to endothelial cells is mediated by cell adhesion molecules that are often modulated by cytokines. Tumor necrosis factor–α (TNF-α) stimulates the surface expression of VCAM on endothelial cells. This TNF-a inducing response in endothelial cells is enhanced by androgen, dihydrotestosterone (DHT). Transforming growth factor–β (TGF-β) stimulates the expression of $\alpha 2\beta 1$ integrin in bone-metastasizing PC-3 cells. In the current study, we investigated the effects of the above cytokines and DHT (singularly and in various combinations) upon HBME cells and prostate cancer cells' expression of VCAM, $\alpha 2$ integrin subunit, and $\beta 1$ integrin subunit by flow cytometry. We also monitored the effects of the above treatments on PC-3 cell adhesion to HBME cell monolayers. The data demonstrates that none of the above treatments significantly altered the expression of selected CAMs on HBME cell and neoplastic prostate cell lines. As a control for TNF-α treatment, human umbilical vein endothelial cells (HUVEC) were treated. VCAM-surface expression was increased by 20 fold on TNF-α treated HUVEC as compared to untreated HUVEC. The treatment of HBME cell monolayers with various combinations of cytokines and DHT prior to performing adhesion assays with PC-3 demonstrates that treatments containing TGF-β reduced PC-3 cell adhesion to HBME cell monolayers by 32% or greater. The reduction in PC-3 cell adhesion to TGF-β-treated HBME cell monolayers was dose dependent. To better access the effect of DHT on prostate cancer cells' ability to adhere to HBME monolayers, we used the androgen-responsive LNCaP cell line. The adhesion of DHT-treated LNCaP cells to HBME monolayers was not significantly different from control LNCaP cells' adhesion. These results suggest that TGF-β may be able to reduce tumor invasion into the bone by regulating CAM expression on bone marrow microvascular endothelium. and DHT, although important for androgen responsive cell growth, is not important for prostate cancer cell adhesion to HBME.

#2313 Repression of Androgen Receptor in Androgen-sensitive LNCaP Prostate Cancer Cells through Inhibition of NF-κB. Heather J. Muenchen, Din-Lii Lin, James D. Gendernalik, Evan T. Keller, and Kenneth J. Pienta. *University of Michigan, Ann Arbor, MI.*

Nuclear Factor-κB (NF-κB) is an inducible transcription factor responsible for the activation of many genes. Its activation is tightly regulated by its endogenous inhibitor, IκB, which complexes NF-κB in the cytoplasm. Phosphorylation and proteolytic degradation of IκB allows the release and nuclear translocation of NF-κB. NF-κB is also a known antagonist of the androgen receptor (AR). Both NF-κB and AR compete for the transcriptional coregulator CREB-binding protein (CBP), which they both require for their transcription. CBP has a greater affinity for NF-κB, therefore, when NF-κB is active, AR is repressed. We have recently demonstrated this phenomenon in the androgen-independent PC3 and DU-145 cell lines, which both constitutively express NF-κB. By using an IκB "super-repressor" (IκBm), which blocks phosphorylation of IκB and subsequent translocation and activation of NF-κB, we have been able to relieve NF-κB's repression of AR in these androgen-independent cell lines. Interestingly, the androgen-sensitive LNCaP cell line, which expresses AR and moderate NF-κB activity, demonstrates repressed AR expression after stable transfection with the IκBm. Both Western blot and RT-PCR analysis of LNCaP cells transfected with the IκBm, demonstrate repressed AR activity with or without R1881 (synthetic androgen). To our knowledge, this is the first case reported where NF-κB and AR behave as agonists rather than antagonists. This repression of AR by blocking NF-κB may be due to mutations, which exist within the AR of LNCaP cells, however, this remains to be explored. The data presented here may give insight as to how prostate cancer progresses from an androgen-dependent to an androgen-independent state.

#674 Functional Androgen Receptor Re-Expression in Androgen-Independent Prostate Cancer Cells. Heather J. Muenchen, Din-Lii Lin, Paul J. Poncza, Lisa L. McLean, Michelle L. Dirette, Evan T. Keller, and Kenneth J. Pienta. *University of Michigan, Ann Arbor, MI.*

Prostate cancer is the second leading cause of cancer-related deaths in men in the United States. The primary treatment for advanced disease is androgen ablation therapy, that is initially effective, but fails to destroy androgen-independent cells, which have a non-functional androgen receptor (AR) and do not require androgen for their survival. These cells continue to multiply causing a relapse in disease. Androgen-independent prostate cancer cells, such as PC3 and DU-145, demonstrate constitutive activation of the transcription factor, nuclear factor-κB (NF-κB), which is a known antagonist of AR. NF-κB is retained in an inactive form in the cytoplasm through association with its inhibitor IκB. Following cellular stimulation, IκB is phosphorylated and degraded, releasing NF-κB, which translocates to the nucleus and initiates transcription of many genes. By using an IκB "super-repressor" (IκBm), which contains alanine substitutions at serines 32 and 36, we have been able to block IκB phosphorylation and therefore, repress NF-κB activation. Western blot analysis of PC3 and DU-145 cells transfected with the IκBm demonstrate re-expression of AR when treated with 10nM of R1881 (synthetic androgen). Enhanced mRNA expression is also detected in PC3 cells stably transfected with the IκBm and treated with R1881. Functionality of the AR was determined by a luciferase assay, which showed that transfected cells demonstrate increased AR activity when treated with 10 nM of R1881. Growth curve analysis of transfected PC3 cells grown in charcoal stripped media and treated with various doses of R1881 demonstrates androgen sensitivity, with a peak response reaching 10^{-7}. The antiandrogen, bicalutamide (Casodex), was able to block proliferation of cells stimulated by R1881. Androgen sensitivity of transfected cells was demonstrated by an *in vivo* growth curve analysis of SCID mice injected with 10×10^6 transfected cells, which demonstrate palpable tumors within 1 week. Control mice did not demonstrate palpable tumors until week 4. Castration of IκBm tumor-bearing mice showed an immediate decrease in tumor volume, whereas castrated control mice continued increasing tumor volume. We demonstrate that repression of NF-κB activity via transfection with the IκB "super-repressor" results in re-expression of functional AR, thereby reversing androgen-independence. These androgen-independent cells are now susceptible to androgen ablation therapy, which will give prostate cancer patients hope for longer and/or permanent remissions.

CELL AND TUMOR BIOLOGY 19: Apoptosis and Therapy I

#2331 Docetaxel Induces Different Apoptotic Pathways in Prostate Cancer Cells LNCaP and PC-3. Heather J. Muenchen, Paul J. Poncza, and Kenneth J. Pienta. *University of Michigan, Ann Arbor, MI.*

In the present study, we investigated the molecular machinery of docetaxel (taxotere)-initiated death signaling on prostate cancer cells, LNCaP and PC-3. Taxotere is a member of the taxane family of chemotherapy agents. It has been shown to disrupt microtubule dynamics causing mitotic arrest, which leads to cell death. Taxotere has demonstrated induction of cell death in LNCaP and PC-3 cells. However, the pathways by which apoptosis occurs differs in each cell line, respectively. Prostate cancer cells, LNCaP and PC-3, were treated with 40 nM of taxotere for various time points (0.5- 24 h). Western blot was used for protein analysis. LNCaP cells demonstrate caspase-3 and –7 cleavage, whereas PC-3 cells show only caspase-8 and BID (BH3- interacting domain death agonist) cleavage. Only LNCaP cells were observed to express clusterin expression while PC-3 cells expressed a novel apoptosis inhibitor, survivin. In this study, we demonstrate two distinctly different taxotere-induced apoptotic pathways in LNCaP and PC-3 cells that may be of clinical importance when treating prostate cancer.

179

RENEWED SENSITIVITY TO ANDROGEN AND ANTI-ANDROGEN IN ANDROGEN-INDEPENDENT PROSTATE CANCER CELLS THROUGH RE-EXPRESSION OF ANDROGEN RECEPTOR BY AN IκB "SUPER-REPRESSOR"

Heather J Muenchen, Din-Lii Lin, Paul J Poncza, Lisa L McLean, Michelle L Dirette, Evan T Keller, Kenneth J Pienta. Ann Arbor, MI*

Introduction and Objectives: Through blocking NF-κB, we propose that androgen receptor (AR) transcription will commence and previously androgen-independent cells will revert to an androgen-sensitive state, leaving them vulnerable to androgen ablation therapy. Androgen ablation therapy for advanced disease is initially effective, but fails to destroy androgen-independent cells, which continue to multiply causing a relapse in disease. Androgen-independent prostate cancer cells, such as PC3 and DU-145, demonstrate constitutive activation of the transcription factor nuclear factor-κB (NF-κB), which is a known antagonist of AR. NF-κB is retained in the cytoplasm by its inhibitor IκB. Following cellular stimulation, IκB is phosphorylated and degraded, releasing NF-κB, which translocates to the nucleus and intitiates transcription of many genes. By using an IκB "super-repressor" (IκBm), we have been able to block IκB phosphorylation and therefore, repress NF-κB activation, causing re-expression of AR.

Methods: AR activity was detected by both Western blot and RT-PCR analysis. A luciferase assay was used to confirm functionality of the AR. *In vitro* growth curve analysis was used to determine renewed sensitivity toward androgen and antiandrogen. Transfected and control cells were treated with varying dosages of R1881 (synthetic androgen) over various time periods. The effectiveness of the IκBm was tested *in vivo* by injecting SCID mice with 10 x 10^6 cells. Mice were then castrated and tumor volume was evaluated.

Results: Western blot analysis of PC3 and DU-145 cells transfected with the IκBm demonstrates re-expression of AR when treated with 10nM of R1881. RT-PCR demonstrates an increase in AR mRNA expression in transfected cells treated with R1881. *In vitro* growth curve analysis of transfected PC3 cells demonstrates renewed sensivity toward androgen, reaching a peak response at 10^{-7}. The antiandrogen bicalutamide reverses this response. *In vivo* analysis demonstrates decreased tumor volume in castrated mice transfected with the IκBm. Control mice continued increasing tumor volume after castration.

Conclusions: We demonstrate that repression of NF-κB activity via transfection with the IκB "super-repressor" results in re-expression of functional AR, thereby reversing androgen-independence.

196

INHIBITION OF NF-κB BLOCKS IGFBP-2 TRANSCRIPTION AND RESULTS IN REPRESSION OF ANDROGEN RECEPTOR IN ANDROGEN-SENSITIVE LNCAP PROSTATE CANCER CELLS

Heather J Muenchen, Din-Lii Lin, James J Gendernalik, Evan T Keller, Kenneth J Pienta. Ann Arbor, MI*

Introduction and Objectives: The mechanism by which prostate cancer cells revert from an androgen-dependent to an androgen-independent or androgen-sensitive state stills remains unanswered. Nuclear Factor-κB (NF-κB) is an inducible transcription factor responsible for the activation of many genes. Its activation is tightly regulated by its endogenous inhibitor, IκB, which complexes NF-κB in the cytoplasm. Phosphorylation and proteolytic degradation of IκB allows the release and nuclear translocation of NF-κB. NF-κB is a known regulator of insulin-like growth factor binding protein-2 (IGFBP-2). IGFBP-2 is one of six binding proteins responsible for allocating IGF-I and IGF-II to various body fluid compartments and tissues and to modulate IGF binding to receptors. IGF-I has been implicated as an androgen receptor (AR) transcription activator in LNCaP cells. By using an IκB "super-repressor", which blocks phosphorylation of IκB, we have successfully blocked NF-κB activation. Blocking NF-κB represses activation of many genes including, IGFBP-2. Without IGFBP-2, IGF-I is unable to bind to its receptor and subsequently activate AR transcription in LNCaP cells. By repressing AR transcription, these formally androgen-sensitive cells are reverted to an androgen-independent state. This data may assist in the effort to explain the transition of prostate cancer cells from an androgen-dependent to an androgen-independent or androgen-sensitive state.

Methods: Western blot analysis was used to determine AR and IGFBP-2 activity of isolated protein from LNCaP cells stably transfected with the IκB "super-repressor" (IκBm). RT-PCR was used to determine AR and IGFBP-2 mRNA activity of transfected cells. A luciferase assay was used to assess functional AR activity in transfected cells.

Results: Western blot analysis of transfected cells demonstrates repression of both AR and IGFBP-2 activity. RT-PCR demonstrates a decrease in the mRNA of transfected cells. The luciferase assay confirmed that AR function is repressed in cells transfected with the IκBm.

Conclusions: To our knowledge, this is the first case reported where AR activity is repressed by blocking NF-κB transcription. This phenomenon is due to inadvertent blocking of IGFBP-2, which requires NF-κB for its transcription. Without available IGFBP-2, IGF-I is unable to bind to its receptor and activate transcription of AR.